NOVEL TOOLS TO IDENTIFY ESTROGEN REGULATED GENES IMPORTANT FOR BREAST AND OVARIAN CANCER CELL PROLIFERATION

BY

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DISSERTATION

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Abstract

Estrogens are steroid hormones produced by the ovaries and extra ovarian tissues including the adrenal gland and adipose tissue. There are 3 major physiologically relevant estrogens in the human body, the most potent and biologically relevant of which is 17β-estradiol (E_2). E_2 exerts its physiologic functions by acting through two isoforms of Estrogen Receptor (ER); ERα and ERβ. ERs are normally bound to heat shock chaperone proteins but once E_2 diffuses across the cell membrane and binds to the ligand binding pocket of ER it sheds these proteins, homodimerizes and binds to DNA recruiting transcriptional machinery and upregulating a number of target genes including those important for proliferation. Estrogens acting through ERα play a central role in the proliferation of breast and ovarian cancer cells as evidenced by the mainstay clinical adjuvant therapies for breast cancer which target the ligand pocket of estrogen receptor by either competitively displacing endogenous ligand, or by inhibiting the rate dependent enzymes responsible for synthesizing the endogenous ligand.

While the importance of E_2-ERα as a central regulator in breast cancer proliferation is unquestioned, it remains unclear how ERα is activated when the clear majority of women who develop breast cancer are post-menopausal. After menopause the concentrations of E_2 are at their lowest point in a woman's life. Epidemiologic studies show that the level of E_2 necessary to increase the risk of breast cancer in a post-menopausal woman is in the picomolar range. However, the known binding affinity of E_2 for ERα lies between 0.5 and 2 nM. It was not known whether E_2 concentrations in the picomolar range can exert biological effects on cell proliferation and gene expression. Moreover, the identity of key regulators of E_2-ERα induced proliferation are not fully characterized. Recent reports indicate that roughly 15% of the human

genome is regulated by Estrogen Receptor. This growing list of ER regulated genes has been compiled, but determining which genes are critical factors in E_2 induced proliferation has remained challenging.

In order to probe these questions, we developed techniques for studying the effect of low concentrations of E_2 on estrogen induced cell proliferation and gene expression. We used this information to begin to identify critical ERα regulated genes. Moreover, we developed a new experimental model that allows us to identify genes critical for E_2-ERα dependent cell proliferation in a nearly isogenic cell system.

Through careful removal of exogenous estrogens we have developed a cell culture system in which the growth of ERα positive cell lines is solely dependent on the addition of E_2 to the system. Therefore, in this system E_2 is the missing factor preventing the cells from proliferating. Using this model we found that picomolar concentrations of E_2 are able to stimulate near maximal proliferation of MCF-7, T47D, T47D ERα D538G, BG1 and PEO4 breast and ovarian cancer cells. Furthermore, picomolar E_2 stimulates robust colony formation in anchorage independent soft agar assays. This effect is ***not*** primarily mediated through ERα's extra-nuclear activation of the ERK signaling pathway, but rather through ERα's classic nuclear mechanism of action. Utilizing qPCR we analyzed expression of a number of genes and find that some genes are concordantly regulated at picomolar and nanomolar concentrations while other genes, such as FOS, were regulated by nanomolar but not picomolar concentrations of E_2. Finally, we show that picomolar concentrations of E_2 are sufficient to elicit robust recruitment of ERα to regulatory elements in estrogen responsive genes.

The second tool described in this work is a set of nearly isogenic cell lines with a specific phenotypic difference. The T47D KBluc cell line was originally developed as a breast cancer cell line stably transfected with a $(ERE)_3$-luciferase reporter in order to detect environmental estrogens. A key difference between this cell line and its parental T47D cell line is that E_2 does not stimulate proliferation of the cells. We demonstrate that this cell line contains ERα, that the ERα is functional and able to regulate both the transfected reporter and endogenous genes. We also tested a panel of known ERα regulated genes to see if any of the genes are discordantly regulated between the two cell lines; this would suggest the gene might play a role in E_2 induced cell proliferation.

These two tools represent an important step in furthering our understanding of E_2-ERα regulated cell proliferation. Notably, we show experimentally that the extremely low concentrations of estrogen identified as tumorigenic in epidemiological studies are sufficient to induce growth of breast cancer cells in the laboratory. This work demonstrates the potential of a novel approach to pursuing genome wide transcriptome studies of ERα action in order to identify critical regulators of ERα induced proliferation.

Acknowledgements

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Chapter One

Background and Significance

1.1 Estrogen concentrations and breast cancer risk

The link between estrogen production and breast cancer has been evident ever since George Beatson described oophorectomy as a treatment for breast cancer in an 1896 edition of the Lancet[1]. Since then the association between early menarche and lifetime estrogen exposure has become well established[2]. However, it is only with more precise measurement and large epidemiological studies that our understanding of postmenopausal hormone levels and the risk of developing cancer has been described.

A 2002 JAMA article demonstrated that the risk of developing breast cancer in post-menopausal women increased 6.9 fold if their serum E_2 levels were greater than 10 pM[3]. A number of more recent articles, while not achieving the same figure, have shown that the serum E_2 levels necessary to increase the risk of breast cancer lie in the low picomolar range[4-7]. This increase in relative risk is despite the fact that the K_d of estrogen receptor is known to be 0.5-2 nM, demonstrating a significant disparity between the known binding affinity of ERα for E_2 and serum E_2 levels[8, 9].

While the links between circulating post-menopausal hormone levels and the risk of developing breast cancer are well described from an epidemiological standpoint, it is unclear whether these hormones levels have measureable biological effects[5]. In fact, it has always been assumed that picomolar concentrations of E_2 were too low to induce gene transcription,

translation of proteins, or carrying out other biological functions in breast cancer cells as well as normal tissues due to the discrepancy between binding affinity and circulating concentrations. In the remainder of this chapter we explore the production of estrogens in relation to normal physiology, its actions through estrogen receptor, its function in breast cancer, as well as its importance as a therapeutic target.

1.2 Estrogen synthesis and transport

Estrogens are a group of steroid hormones derived from cholesterol that are produced by various tissues in the human body including the ovaries, adrenal glands, and adipose tissue (Fig 1.1)[10]. Estrogens, as a general class of steroid hormone, are the endogenous ligands for both the α and β isoforms of estrogen receptors with Estrogen Receptor α (ERα) being the major mediator in breast cancers. There are three endogenous estrogens; 17β-estradiol (E_2), estrone (E1) and estriol which have physiologically relevant effects [11-13] However, the hormone with the highest binding affinity for ERα and the greatest biological significance, especially related to breast cancer, is 17β-estradiol (E_2)[8] .

During the ovulatory years of a woman's life estrogen production in the human body is a highly regulated endocrine process requiring multiple levels of signaling. This system requires the hypothalamus, pituitary, and gonads to function in concert to produce estrogens. The ovaries are the major site of Estradiol production in the pre-menopausal woman. The two-cell theory for estrogen production as outlined in Figure 1.2 illustrates the basic mechanism of action[14]. GnRH in the hypothalamus is released in to the hypothalamic portal system where it acts on the posterior pituitary to stimulate the secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) from gonadotropes in the pituitary. From the pituitary LH and FSH

travel via the blood to act on the ovaries. Two distinct cell types within the ovarian follicle epithelium participate in the coordinated production of 17β-estradiol; theca cells and granulosa cells. Theca cells are acted upon by LH through the Luteinizing Hormone Receptor to promote the conversion of cholesterol via a multistep pathway to androstenedione.

As a hydrophobic steroid hormone, androstenedione can diffuse through cell membranes in to the neighboring granulosa cells where the androstenedione is converted to estrone (E1). This takes place through a member of the cytochrome P450 family of enzymes CYP19A1, a monoxygenase, also known as aromatase. The activity of aromatase is regulated by FSH acting via the FSH receptor on granulosa cells. A second enzyme 17 Beta hydroxysteroid dehydrogenase (17β HSD1) reduces estrone (E1) to estradiol (E2). From the ovaries E2 is able to diffuse across cell membranes and travel within the blood predominantly bound to albumin and sex hormone binding globulin (SHBG) while less than 2% remains unbound. From the blood estradiol can exert its effects on many tissues including the uterus, breast, bone, and brain [12-17].

The site and amount of estrogen production, particularly estradiol, varies from the premenopausal state to the postmenopausal state. As outlined above, estrogen production in the premenopausal woman takes place primarily in the ovaries. However, during menopause ovarian senescence eventually leads to amenorrhea and a significant decrease in gonadal estrogen production. Consequently, in the post-menopausal woman the majority of estrogen production takes place in extra ovarian tissue such as the adrenal cortex[18]. The major estrogen secreted by the adrenal cortex is in the form of estrone which can be converted by aromatase in peripheral adipose tissue to estradiol[13, 19]. Therefore, the circulating levels of E_2 tend to be higher in obese individuals with large amounts of adipose tissue than those who do not have an elevated

BMI [20-23]. However, the result is a precipitous decline in overall estrogen production as well as a drop in E_2 production due to the lack of conversion of E_1 to E_2 [15, 16, 24] . Figure 1.3 diagrams variation in E_2 levels over a woman's lifetime[25]. The figure illustrates that E_2 levels increase from puberty, peak during menstrual cycle and drop to some of the lowest points during menopause. Estimates vary regarding the differences in circulating concentration before and after menopause. However, there is general agreement that circulating concentrations of E_2 drop from peak ovulatory levels in the nanomolar range to the picomolar range[4, 6, 7, 26]. Best estimates place the lowest levels of estradiol during normal menstrual cycle at approximately 200 pM while the highest circulating levels are approximately 1500 pM. After menopause the circulating levels of estradiol have a wide range of estimates from 1-15 pM [27].

1.3 ERα structure and action

Estrogen Receptor (ER) is a Class I member of the nuclear receptor superfamily that binds and is activated by the endogenous ligand 17β-estradiol (E_2). In the human body there are two isoforms of Estrogen Receptor, α and β, which are encoded by *ESR1* and *ESR2* on distinct chromosomes. While both isoforms are expressed in a wide variety of tissues and are important physiologically, ERα is more directly implicated in oncogenesis. Structurally ERα has 12 α helices and a number of characterized functional domains (Figure 1.4) that contribute to its actions. Notably it has a ligand binding domain, a DNA binding domain (DBD), and 2 activation function domains (AF 1 and AF2)[28-30].

Classically, ER function is described as regulating the transcription of genes via interactions with DNA. The majority of ERα is found in the nucleus bound to stabilizing heat shock proteins such as HSP90. Binding of E_2 to ERα releases E_2-ER complex from stabilizing

proteins and allows the activated receptor to homodizmerize[31]. Activation and homodimerization allows ER to bind via zinc fingers in the DBD to DNA sequences called Estrogen Response Elements (EREs) found either at the promoter or at distant sites several thousand base pairs away called enhancers (Fig 1.5)[32]. Once bound to DNA ER can recruit co-activators and co-repressors through the activation function domains to assemble a transcription complex and regulate the transcription of target genes. Specifically, helix 12 appears to be of critical importance to transcriptional regulation through the recruitment of co-activators, such as the p160 family of proteins or co-repressors including NcoR and SMRT which utilize an LXXLL motif. Alternatively, the receptor can induce transcription of genes through tethering mechanisms that do not require direct interaction of the E_2•ER complex with DNA at AP-1, SP-1, RUNX1 and FOXA1 binding sites [33-36] .

The classical mechanism description of ER action revolves around its originally recognized role as a transcription factor. However, in recent years alternate mechanisms of ERα action have gained broad acceptance. The most prominent and best studied to date is non-genomic ERα signaling, where membrane bound receptor activates rapid signaling pathways such as MAP kinase and Akt. Since ERα does not have a catalytic domain, the activation of these signaling cascades relies on the formation of multiprotein complexes around ERα resulting in the activation of signaling cascades [37, 38].

Another mechanism of action proposed for estrogens is via a unique orphan G Protein Coupled Receptor (GPCR) named GPR30. GPR30 is a GPCR that has been reported to bind E_2 with high affinity[39-41]. GPR30 has been reported to function by activating intracellular signaling mechanisms including cAMP and the kinase PKA resulting in the regulation of ion channels and fluxes in intracellular or intraluminal calcium[42, 43]. However, the

characterization of GPR30 is not without a number of conflicting reports including the relative concentrations necessary to activate the receptor which have been reported as greater than 1 uM; reports that very high concentrations anti estrogens such as 4-OHT and Fulvestrant activate the receptor; that anti-estrogens have been reported to increase the proliferation of breast cancer cells; and even the subcellular localization of the receptor [44, 45]

Recently, the importance of ERα in regulating the rate of protein synthesis by activating the Unfolded Protein Response (UPR) has been reported[46]. As part of the process for initiating proliferation of breast cancer cells the endoplasmic reticulum must balance the synthesis of new proteins with the availability of chaperones which is directed by the UPR. Recent studies have revealed that ERα is able to regulate this pathway via activation of PLCγ which causes a calcium efflux from endoplasmic reticulum resulting in a temporary halt to translation via the phosphorylation of eIF2α and readying of the chaperones via the remaining two arms of the UPR for correct protein folding [46-50].

Estrogens, most importantly E_2, operate via several pathways to regulate the proliferation of cells. These pathways utilize ERα primarily to regulate gene transcription and cell proliferation.

1.4 The estrogen transcriptome and key players

A key question in the estrogen receptor field has been the identity of the genes ERα regulates. Identification of ERα regulated genes has proved exceedingly difficult since ERα binding sites have diverse sequences and, through enhancer elements, can be thousands of base pairs away from the gene they regulate[51, 52]. Also, indirect ER interaction with DNA through protein-protein tethering mechanisms make the identification of genes difficult. Recent papers

have suggested that as much of 15% of the global transcriptome could be regulated by E_2 through ERα[53]. While the global regulation of a significant portion of the genome through the E_2-ERα complex is true at hormone concentrations several fold higher than is seen at ovulation it is unknown whether these effects are evident at the lower physiologic concentrations observed in post-menopausal women.

It is known that ERα regulates a number of putative cell cycle genes important in the proliferation of breast cancer cells including E2F1, c-fos, and c-myc[54-58]. ERα also has a role in inhibiting apoptosis through factors such as BCL-2 and BCL-XL[34, 59]. However, the list of genes tested spans tens of genes and distinguishing between key players regulated by E_2-ERα through direct transcriptional mechanisms and secondary actors not directly regulated by estrogen receptor has been difficult.

1.5 Estrogens and estrogen receptor in normal physiology

In non-pathologic physiology E_2 and ERα have important roles to the growth and development of a wide variety of tissues in both men and women. These tissues include but are not limited to brain, bone, breast, uterus, and cardiovascular systems [11, 13, 15, 17]. Below we shall briefly discuss a few tissues in which the importance of E_2-ERα have been well established.

Bone is a tissue in which estrogens have been shown to play an important role even though only a few hundred molecules of ERα are present in a given bone cell[60-62]. In bone estrogens such as E_2 modulate the closure of epiphyseal plates, bone maturation and, most importantly, bone resorption[17]. Estrogen has been shown to modulate osteoclast and osteoblast activity. This apparent through pathologic conditions such as osteoporosis where one of the mainstay treatments for women is raloxifene, an estrogen receptor modulator[63, 64].

E_2-ERα's role in the cardiovascular system is complex owing to the number of factors that influence cardiovascular health including lipid metabolism, hypertension, diabetes and other comorbid conditions. Much of what is concluded about the influence of estrogens is based on large scale epidemiological data with correlation through animal models. E_2 influences lipid metabolism through the liver. Estrogen increases HDL and lowers LDL which are markers for cardiovascular disease. Evidence suggests that E_2 has a protective role in cardiovascular health as evidenced by the increase in stroke and myocardial infarction after surgical or physiologic menopause. While the Women's Health Initiative Study (WHI) could not demonstrate a decrease in cardiovascular disease with Hormone Replacement Therapy there were indications within the study group that HRT at the onset of menopause may be related to lower CVD risk. However, subsequent studies analyzing this timing hypothesis have been unable to prove a beneficial effect of early initiation of HRT [65].

Estrogen exerts its influence on breast development at puberty during a period known as thelarche which is usually between the ages of 8-13[66]. Breast tissue is made up of between 12-20 conical lobes each of which consists of a group of lobules that contain lactiferous ducts. At puberty, in response to E_2 stimulation, breast tissue starts to enlarge owing to an increase in acinar tissue, ductal epithelial proliferation, and subsequent increases in amount of adipose tissue. While E_2-ERα interactions are important to the normal growth and development of breast tissue, later in life pathologic mutations in the breast epithelium contribute to breast cancers in which estrogen and estrogen receptor are key players [11, 17, 65].

1.6 Importance of ERα in breast cancer

Breast cancer is defined as a carcinoma normally found through the clinical presence of a firm immobile mass in the breast tissue or, in the United States and other developed countries, through routine screening mammography[65]. Breast cancer is the third leading cause of cancer deaths in the United States. Approximately 225,000 women per year will develop breast cancer and almost 40,000 women will die, meaning that about 1 in 8 women will be diagnosed over their lifetime[67, 68]. Men are also at risk of developing breast cancer but they account for less than 1% of all cases[67]. The risk of developing breast cancer rises with age and increases dramatically after age 50. The risk of developing breast cancer at 40 is approximately 1: 250 and rises to 1:50 by age 50[17].

Most breast cancers (>95%) arise from the breast epithelium. Clinically the diagnosis of breast cancer must be made from histologic makeup of the cells derived from a biopsy of the mass. However, once a histologic diagnosis has been made based on the sample architecture and cell morphology, molecular diagnostics can be used to further subdivide the breast cancers and provide information on prognosis and treatment. Receptor testing for the presence of ER and PR, defined as positive immunohistochemistry >1% of the histologic sample, is a mainstay of classifying prognosis and treatment. In a sample of 61,309 cases of breast cancer roughly 80% were ER+ and/or PR+, 23% were HER2+, while 13% were negative for all three [69]. Therefore, $ER\alpha^+$ breast cancer is the most prevalent type of breast cancer, accounting for nearly 70% of the diagnosed breast cancer cases with increased incidence due to age[70, 71].

Once a diagnosis has been established surgical excision of the cancer via either a lumpectomy, removal of the local tumor tissue along with a margin for healthy tissue, or

modified radical mastectomy are undertaken [72, 73]. Adjuvant therapy added on to the treatment regimen after surgery can also be undertaken. This could consist of chemotherapy regimens, radiotherapies, or endocrine therapies [74]. The decision of which treatment regimen to follow relies on immunohistochemical analysis of the sample. In the case of breast cancer, the presence of the hormone receptors ER and PR predicts the response to endocrine therapies which target either estrogen receptor or the ligand 17β estradiol. The degree of endocrine therapy benefit is directly related to the level of ERα expression within the tumors. A meta-analysis of the Early Breast Cancer Trialists Collaborative Group showed the response to adjuvant therapy with tamoxifen reduced the recurrence by 39% and death by 30% in 15 year follow up[75].

Besides the information provided by histology and immunohistochemical studies molecular information also provides information on prognosis. Staging of breast cancer based on tumor burden and the presence of distant metastasis provides the best information on prognosis but molecular information based on gene expression profiles from biopsy samples can provide a wealth of information. The three major molecular subtypes are luminal, Human Epidermal Growth Factor Receptor 2 (HER2) enriched, and basal subtypes. Luminal A and B subtypes are characterized by similarity in gene expression between the cancer and the luminal epithelium [59, 69, 76-81]. Luminal A have high expression of ER related genes, low expression of HER2, and low expression of proliferation related genes. Luminal B have lower expression of ER related genes and high expression of proliferation related genes. The HER2 overexpress a type of human epidermal growth factor receptor which has a targeted therapy called Herceptin. Basal subtypes, named for the gene expression similar to basal epithelial cells, are ER negative and generally fall under the category of "triple negative" breast cancers. Triple negative cancers have a poor prognosis.

The importance of estrogen receptor in breast cancer is evidenced by the success of therapies that target it. Mainstay adjuvant therapies for $ER\alpha^+$ breast cancer target the receptor via 2 broad modes of action. The first involves inhibiting the aromatase enzyme thereby suppressing E_2 production and depriving ER of its endogenous ligand. The second method involves blocking the binding of E_2 to ERα by utilizing competitive inhibitors. Tamoxifen a Selective Estrogen Receptor Modulators (SERMs) and Fulvestrant a Selective Estrogen Receptor Degraders (SERDs) compete with E2 for the Ligand Binding Domain (LBD) of ER[29, 82, 83].

1.7 Endocrine therapies in the treatment of breast cancer

ERα is an important therapeutic target in breast cancer as evidenced by the number of tumors that express the receptor, the receptors' role in promoting proliferation and tumorgenesis, as well as its clinical importance in the prognosis of breast cancer. There are two major therapeutic approaches to targeting estrogen receptor. The first is to target ERα via its classical mechanisms of gene transcription through pure or partial antagonism of the receptor by displacing the endogenous ligand. The second is to target E_2 the ligand that binds and activates ERα by decreasing its production from ovarian or extra ovarian tissue.

One of the most successful medications in the treatment of breast cancer over the last 50 years has been the SERM tamoxifen. Tamoxifen is structurally similar to estrogen, as seen in Figure 1.6 [20]. Tamoxifen is able to block the functions of estrogen receptor in breast tissue by binding to the ligand binding pocket and displacing helix 12. Helix 12 normally functions to recruit co-activators but its displacement blocks the interaction surface for coactivators[84, 85]. While tamoxifen acts as an antagonist in the breast, it functions as a partial agonist in bone,

endometrial, and cardiovascular tissue presumably because of difference in coactivator expression within these tissues[29].

Another type of competitive inhibitor which is considered a third line endocrine therapy is called ICI 182 780, also known by the trade name Fulvestrant or Faslodex[82]. Unlike tamoxifen, ICI is not a SERM but a SERD which does not have any partial agonist activity and functions as a pure antagonist. Fulvestrant is also similar in structure to endogenous estrogens (Figure 1.6)[20]. It functions by binding to the ligand binding pocket but induces degradation of the protein through the 26s proteasome [82]. Two major drawbacks to the clinical utility of Fulvestrant are reports that it can take up to 6 months to reach therapeutic levels in the blood as well as the fact that for long term therapy tissue specific antagonism is preferable due to the beneficial effects of estrogen on the bone and brain [63, 64].

A second category of clinically important therapies are aromatase inhibitors (AIs) [86]. Aromatase is the rate limiting enzyme in the biosynthesis of 17β Estrasdiol and the enzyme is overexpressed in many breast cancers[87]. Two types of AIs are used clinically, Type I and Type II, both of which block the production of E_2. Type I AIs are structurally similar to androsetendione, which is a natural substrate for aromatase. The enzyme acts on the Type I AI and produces an irreversible covalent bond which prevents the synthesis of E2. Type II AIs on the other hand do not use an irreversible mechanism of action. Rather, Type II AIs are competitive inhibitors which do not resemble steroid molecules. They can be displaced with an increase in androgen synthesis. The complete mechanism by which Type II inhibitors work is not know but it has been show that they functionally inactivate a heme group within the enzyme[13]. Both Type I and Type II AIs are used after 5 years of tamoxifen treatment and are useful in the treatment of tamoxifen resistant breast cancers[88]. However, because of their systemic effects

on estrogen production they have an unfavorable side effect profile and are considered later in therapeutic progression.

The ability to target a singular receptor through multiple mechanisms of action evidences the importance of ERα as a key factor in the growth and progression of breast cancer. The mechanisms of targeting estrogen receptor also highlight the importance of displacing the endogenous ligand. Both major classes of clinically relevant endocrine therapy block the action of E_2 with relation to estrogen receptor as much as they block the action of ER itself.

1.8 Current work

In the following chapters we describe current work to answer key questions in the E_2-ERα field. Our efforts have led to the development of assays that allow us to study the direct biological effects of ERα *in vitro* using concentrations of E_2 similar to those seen in post-menopausal women. We find that 5 pM E_2 promotes the growth of breast and ovarian cancer cells. These assays have also allowed us to study the effect of low E_2 on the various pathways of ERα action including the extra nuclear and genomic pathways. We have been able to study the effect on low concentrations of E_2 on gene transcription in order to study differences in gene regulation of putative ERα target genes. While these studies have shed light on a number of issues related to ERα and estrogen concentrations, they have also opened up new avenues of research on estrogen receptor dynamics at sub-saturating ERα concentrations that were previously considered to be technically unfeasible.

1.9 Figures

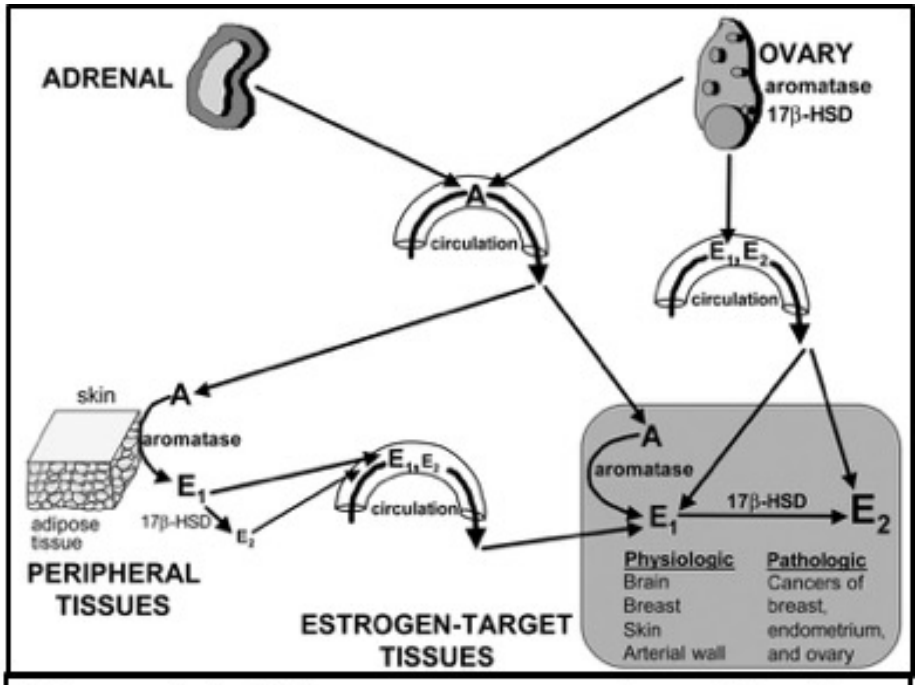

Figure 1.1 Estrogens are produced by the ovary, adrenal gland, and adipose tissue.

Adapted from Bulun et al. (2007) Aromatase Excess in Cancers of Breast, Endometrium, and Ovary. J Steroid Biochem Mol Biol. 106 (1-5): 81-96

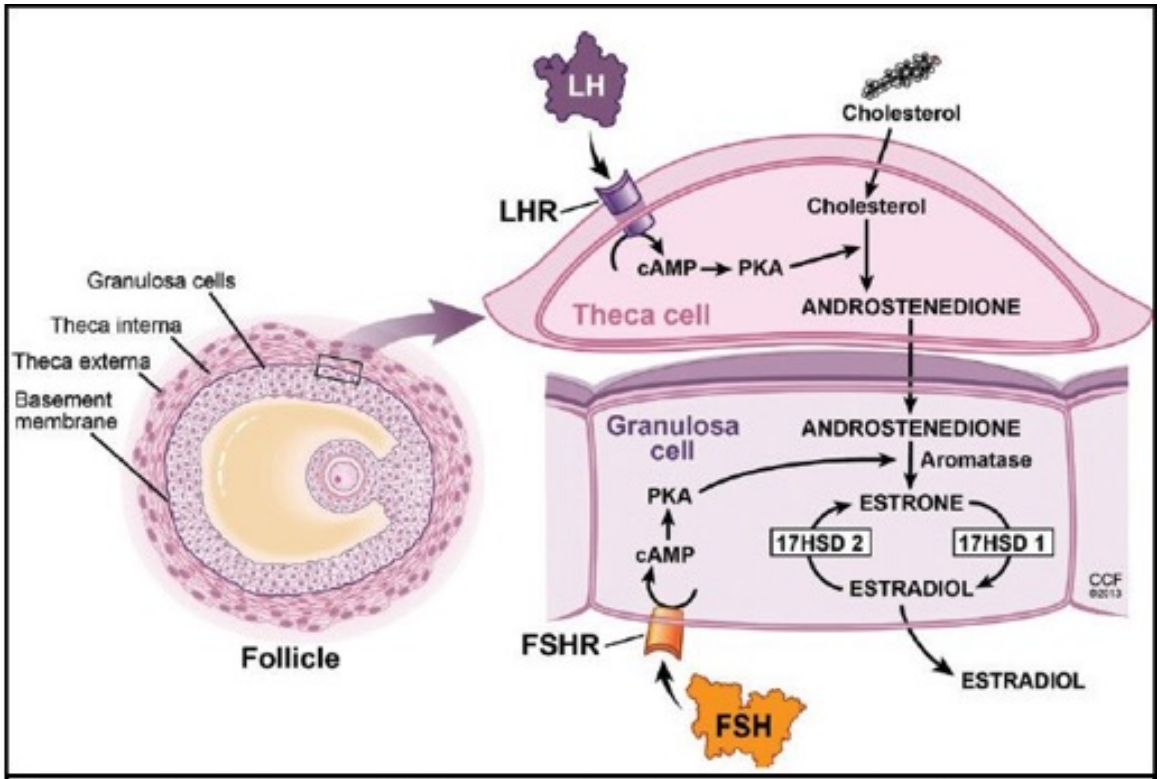

Figure 1.2 Two cell theory of estrogen production.

Adapted from Doshi and Agarwal (2013) The Role of Oxidative Stress in Menopause, J Midlife Health 4 (3): 140-6).

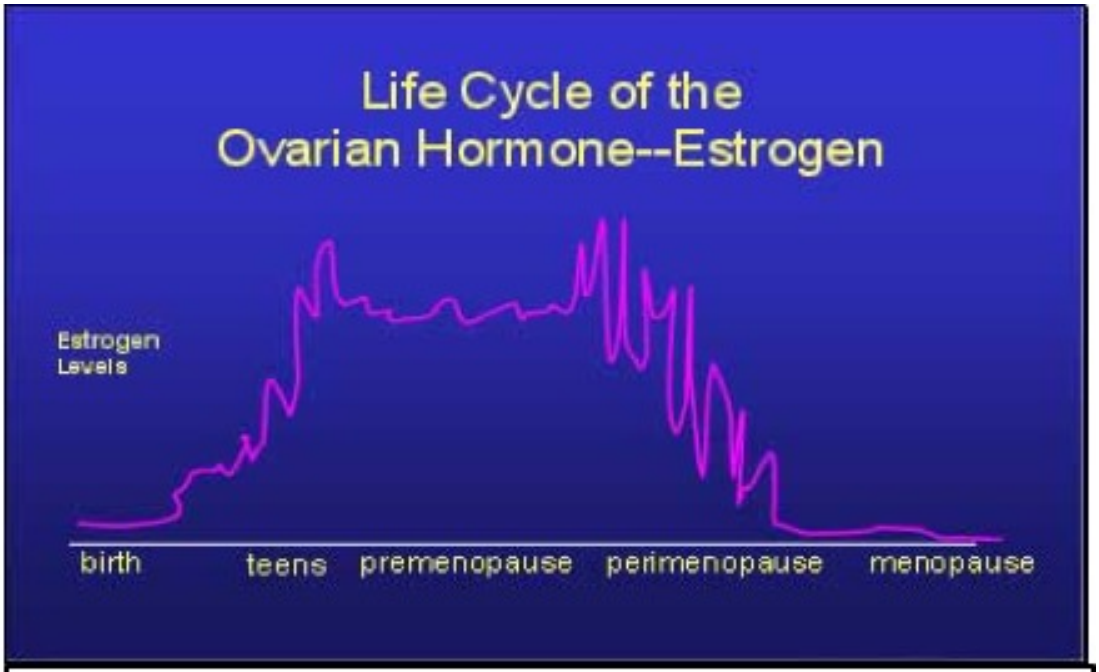

Figure 1.3 Estrogen production over a women's lifetime.

Adapted from JC Prior (2007) Centre for Menstrual Cycle and Ovulation Research.

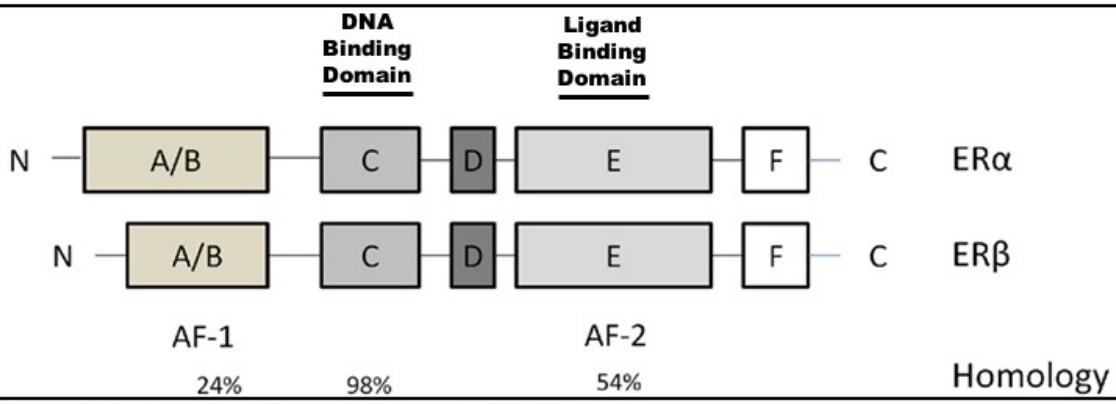

Figure 1.4 The structure of ERα and ERβ domains.

Adapted and modified from Karimian et al. (2011) Genetic Regulation of the Growth Plate. Front Endocrinol. 2:113

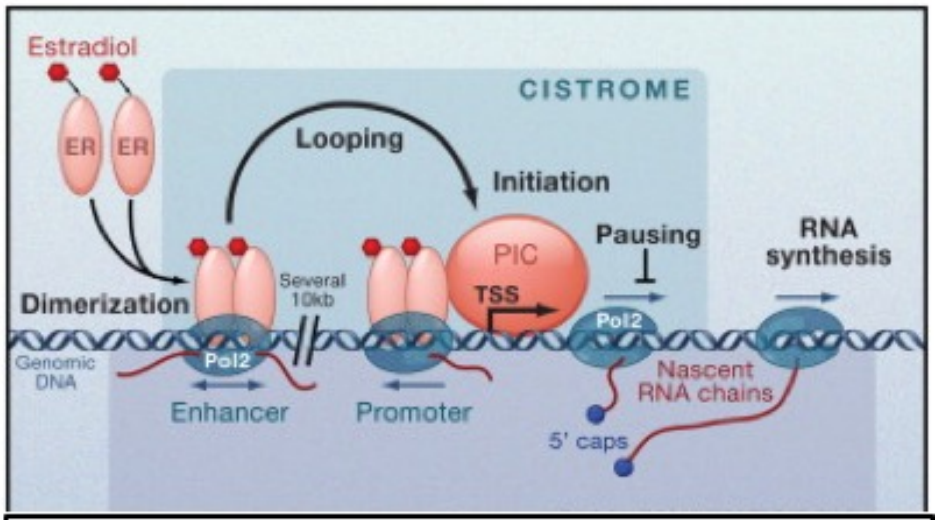

Figure 1.5 Estrogen Receptor α regulates transcription.

Adapted from A. Prokesch and MA Lazar (2011) A Hormone Sends Instant Messages to the Genome. Cell. 145:4 499-501

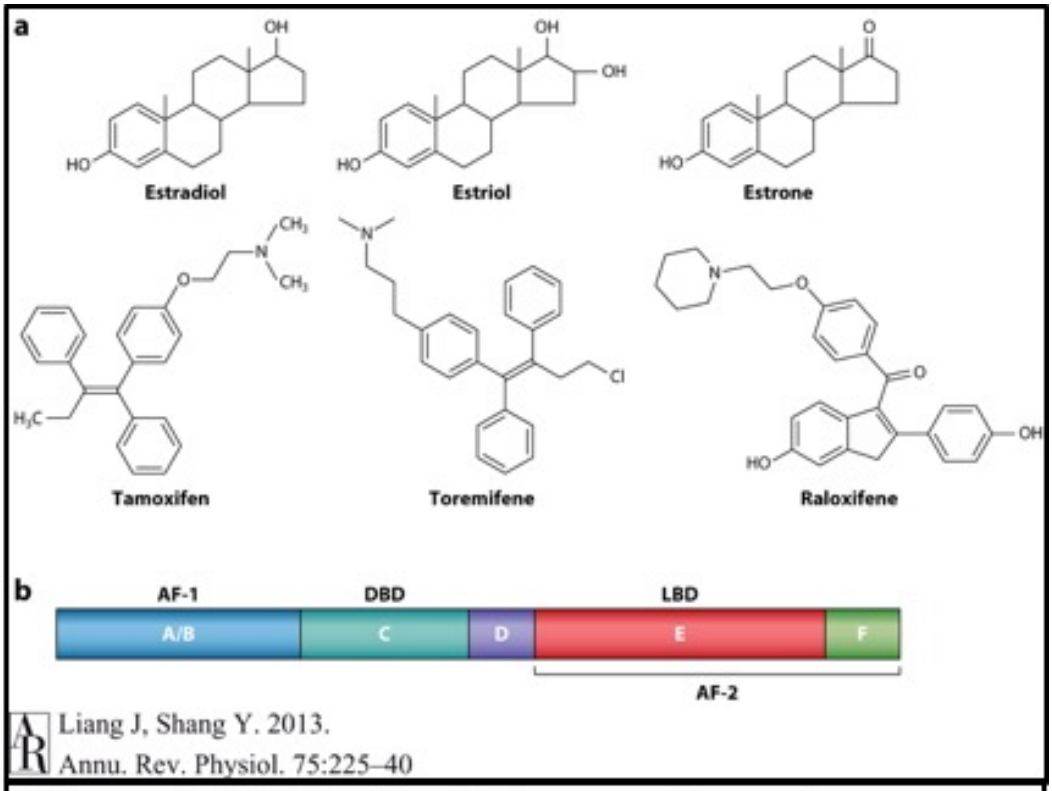

Figure 1.6 Selective Estrogen Receptor Modulators are similar in structure to endogenous estrogens.

Adapted from Liang et al. (2013) Estrogen and Cancer. Annual Review of Physiology. Volume 75: 225-40

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Chapter Two

Physiologic Concentrations of 17β-Estradiol Induce Cell Proliferation via Estrogen Receptor α

2.1 Abstract

Estrogen Receptor (ER) plays an important role in the growth and progression of breast cancer and, as evidenced by the success of tamoxifen, an important adjuvant therpay. The clear majority of ER positive breast cancers occur in postmenopausal women when 17β-estradiol (E_2) levels are at their nadir. Epidemiological studies have established that the level of E_2 necessary to increase the risk of a woman developing breast cancer is much lower than the reported K_d of estrogen receptor and lies within the picomolar (pM) range. We show that *in-vitro* the levels of estrogen in concordance with those seen in postmenopausal women elicit robust proliferation of several breast cancer cell lines in both 2D and 3D cell culture models. Using pharmacological inhibitors of ER and siRNA knockdown, we demonstrate that these effects are mediated through ERα. We also demonstrate that these effects are primarily mediated through estrogen receptor's nuclear effects and are not via ER's extra-nuclear activation of ERK. These results indicate that only a very small fraction of the total receptor population needs to be ligand bound in order to induce near-maximal proliferation of ERα positive cancer cells.

2.2 Introduction

Breast cancer is the second leading cancer in the United States with almost 40,000 new cases annually[1]. Two key players in the growth and progression of breast cancer are estrogens and Estrogen Receptor (ER)[2, 3].

Estrogens normally produced by the ovary and adrenal gland, act via estrogen receptor and play a critical role in the development and progression of breast cancer[3, 4]. The endogenous estrogen, 17β-estradiol (E_2), binds ERα and creates an E_2-ERα complex. This complex exerts effects directly on the genome as well as through extra-nuclear effects which activate intracellular signaling cascades both of which ultimately promote the proliferation of breast cancer cells[5].

The classic pathway of E_2-ERα action is via the nuclear pathway. When E_2 binds to ERα, localized in the nucleus, this causes dissociation of heat shock proteins from the unlignaded receptor and recruitment of ERα to form DNA-bound transcriptional complexes. ERα can bind directly to DNA at palindromic sites termed Estrogen Response Elements (ERE), or it can bind to basal transcription factors such as the AP-1, SP-1 or FOXA1 family of transcription factors in a mechanism known as tethering[6-10]. Ultimately this leads to transcription of ER target genes important for several biological functions including proliferation.

The second method by which the E_2-ERα complex exerts its influence on the cell is via the extra-nuclear or rapid pathway. Here E_2 bound ERα interacts with a multitude of signaling cascades outside the nucleus to quickly initiate signaling events[11, 12]. The classic extra-nuclear target of E_2-ERα is the activation of the ERK 1/2 pathway which promotes phosphorylation of transcription factors and downstream MAPK targets[13].

Despite our understanding of the various pathways E_2 regulates via ER we still don't understand how activation of those pathways may vary over the life time of a woman. The vast majority of women who develop ER^+ breast cancer are postmenopausal[14]. This is when circulating estrogen concentrations are at their lowest. Improved methods for detecting and quantitating circulating estradiol levels in blood have led to several papers which highlight the surprising finding that picomolar levels of circulating estrogens increase the risk of breast cancer [15-19]. Most prominently a JAMA article demonstrated that compared to undetectable levels (<1 pM), a serum estradiol level of 10 pM increased the risk of developing breast cancer by 6.9 fold[20].

While the epidemiologic data is robust and compelling, it is not clear whether these levels of E_2 can induce proliferation of breast cancer cells, or merely increase the risk of developing breast cancer. In order to assess this, we developed a cell culture based system in which the cells are completely dependent upon estrogens for their growth. Using this assay we could then test whether concentrations of E_2 well below the K_d for binding to ER (~0.5 nM) could stimulate cell proliferation[21, 22].

2.3 Materials and Methods

Cell culture

Cells were maintained in MEM supplemented with 10 mM HEPES and either 5% (MCF-7 and BG-1) or 10% (T47D) FBS. 4 days prior to plating cells were cultured in MEM supplemented with 5% (MCF-7 and BG-1) or 10% charcoal-dextran-treated (CD)-FBS.

Cell proliferation assay

Cells were cultured in charcoal dextran stripped media as described above. Cells were trypsinized and re-suspended in 10% CD Calf Serum. Cells were plated in a 96-well plate at a density of 1,000 cells/well (MCF-7), 2,000 cells/well (T47D and T47D D538G) or 200 cells/well (BG-1). After 12 hours the plating media was removed and replaced with media containing the desired treatment in 10% CD-CS . After 3-4 days the cells were incubated with Cell Titer 96 Aqueous One Solution (Promega) (MTS assay) and analyzed using a spectrophotometric plate reader at 490 nM and 650 nM. A standard curve was always performed to correlate cell number to the absorbance reading. Proliferation was confirmed by visual observation of each well.

Soft agar colony formation assay

To assay anchorage-independent cell growth in soft agar, 1 and 0.7% Select Agar (Invitrogen) was prepared in water and warmed at 40° C before use. 1.5 ml of 0.5% bottom agar, diluted in 2× MEM, was added to each well of a 6-well cell culture plate and allowed to solidify at room temperature. Top agar was prepared by dilution in warm medium. MCF-7 cells were resuspended in 1.5 ml of 0.35% top agar at 6,000 cells/well and plated in 3 wells for each condition. The plates were kept at room temperature for 30 min until the top agar solidified, then 0.5 ml of medium containing the respective treatments was added on top of the agar. Culture medium containing the treatments were changed every 2-days. Colonies were visible after 2 weeks in the hormone-treated wells and counted at day 28 using a dissecting microscope. Photographs of colonies ERα in each cell line were taken using a Zeiss AxioImager2 imaging system at 5× magnification.

Transfection

25,000 MCF-7 cells were plated in antibiotic free 5% CD-FBS in a 24-well plate. The cells were allowed to adhere overnight. The cells were treated with 5 nM Faslodex/ICI 182,780 (Sigma) for 12 hours before initiation of the transfection protocol. Cells were treated with 50 nM of ERα siRNA, 50 ng of scrambled siRNA or liposome alone. Cells were treated with transfection complex for 16 hours after which the complex was removed and replaced with antibiotic-free10% CD-CS. Cells were then plated in 96-well plates for treatment and analysis of cell proliferation/MTS assay alongside a standard curve.

Western blot analysis

Cells were cultured in 5% CD-FBS (MCF7 and BG-1) or 10% CD-FBS (T47D) for 4 days prior to being plated at 300,000 cells/well in 6-well plates in medium containing 10% CD-CS. Whole cell extracts were prepared after 24 h of treatment using 1× radioimmune precipitation assay buffer (Millipore) containing complete mini protease inhibitor mixture (Roche). 30 μg of protein per lane was analyzed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). Phospho0p44/42 MAPK (4370) p44/42 MAPK (4695), ERα was detected using Santa Cruz (8002) monoclonal antibody T1699 (Sigma), and β-actin was detected using antibody A1978 (Sigma).

Statistical analysis

All statistical analysis was performed using two-way ANOVA with Bonferroni correction.

2.4 Results

Picomolar levels of 17β-estradiol induce proliferation of breast and ovarian cancer cells

In a mouse xenograft model for breast cancer, MCF-7 tumors exhibit complete dependence on the presence of estrogen for growth[23-25]. In contrast, under standard cell culture conditions, MCF-7 cells grown in the absence of added E_2 actively divide and simply grow more rapidly after treatment with E_2[25] . Studies performed in dozens of laboratories over the last 30 years reveal a wide range of dose-response curves that have been reported for E_2 stimulation of the growth of MCF-7 cells[26, 27]. I, and others in our laboratory, developed a number of techniques to more nearly simulate breast cancer proliferation conditions in the intact animal and eliminate traces of estrogens from the culture system. In my experiments, growth of MCF-7 cells is completely dependent on addition of E_2 to the culture medium. I reproducibly find that concentrations of E_2 in the low picomolar range stimulated growth of the MCF-7 cells. Half-maximal stimulation of growth was achieved at ~0.5 pM E_2 and maximal stimulation of MCF-7 growth was achieved at 5 pM E_2 (Fig. 2.1 A).

To test whether the surprising finding that picomolar E_2 stimulates growth of MCF-7 cells reflects a unique property of these cells, or is a more general phenomenon, I performed dose-response studies of the effects E_2 on the growth of T47D human breast cancer cells, the BG-1 variant of breast cancer cells and a mutant T47D D538G cells derived from wildtype T47D cells. Maximal cell proliferation was achieved at 10 pM E_2 for T47D cells, nearly 10 pM E_2 for T47D D538G cells and 20 pM E_2 for BG1 cells growth was achieved while maximal proliferation (Figure 2.1 B, C, D). While the MCF-7, T47D and BG1 cells did not demonstrate estrogen independent growth the T47D D538G cells did proliferate in the absence of E_2.

While the importance of estrogens and estrogen receptor are well described in breast cancer due to the efficacy of endocrine therapies, ovarian cancers represent another often-overlooked area where the E_2-ERα signaling pathway are important. 30-70% of ovarian tumors are ER^+, rely on the expression of estrogen receptor but are insensitive to endocrine therapies[28-30]. PEO4 cells are one model of ovarian cancer that are known to estrogen responsive in cell culture [31]. PE04 cells plated in our cell culture system demonstrate near maximal estrogen inducible growth at 50 pM (Fig. 2.1 E). The resulting data demonstrates that both breast and ovarian cancer cells are induced to near maximal proliferation by picomolar concentrations of E_2.

Estrogen receptor levels correlate with the concentration of E_2 required to stimulate proliferation

In each cell line pM estrogen elicited robust proliferation. To test whether differences in E_2 concentration required to induce maximal stimulation of cell proliferation was related to the level of ERα, we determined the level of ERα in each cell line. Figure 2.2 shows that receptor levels in the T47D and BG1 cells are roughly half and one third, respectively, of the ERα level in MCF7 cells. The T47D D538G derived from the wildtype T47D cells have been shown to have a similar level of ER[32]. Thus, ERα levels show an inverse correlation with the concentration of E_2 necessary to induce maximal proliferation. The results also correlate with the fact that approximately twice the amount of estrogen was necessary for maximal proliferation of the T47D cells compared to the MCF-7 cells.

Picomolar estrogen stimulate anchorage independent growth

While 2D cell culture assays are robust tools for studying the division of cells they cannot completely recapitulate all the properties of a 3-dimensional tumor. An *in vitro* surrogate of

tumor xenografts are soft agar or colony formation assays. These 3-dimensional cultures recapitulate 2 essential features of cancer cells. The first is anchorage independent growth of tumor cells while the second is clonal outgrowth of a single tumor cell[33, 34].

In order to provide a more rigorous and physiologic test of the ability of pM E_2 to induce cell proliferation, we performed soft agar assays (Figure 2.3). Using no estrogen (-E_2), 10 nM E_2 and 10 pM E_2 we observed that pM concentrations of E_2 stimulate the formation of colonies >0.5 mm in diameter. Comparing the number of colonies >0.5 mm between treatment groups, there was a statistically significant ($P<0.05$) difference between the -E_2 and either the pM E_2 or nM E_2 groups (Figure 2.3 C). However, there was not a significant difference in the number of colonies formed between the groups treated with pM and nM estrogen. This demonstrates that low concentrations of estrogen in the physiological range of postmenopausal women promotes anchorage independent growth of breast cancer cells.

Picomolar estradiol functions via ERα

To explore whether the effects of pM E_2 were mediated through ERα, or via a new novel mechanism of action, we inhibited estrogen dependent growth utilizing the competitor antiestrogen ICI 182,780/Faslodex/fulvestrant and our novel non-competitive small molecule ERα inhibitor, TPSF[35, 36]. TPSF is a specific small molecule inhibitor of ER alpha identified via *in vitro* screening that works outside the ligand binding pocket of ERα. This is in contrast to ICI 182780/Faslodex/fulvestrant which competes with estrogens for the ligand binding pocket of ERα [37, 38]. These two inhibitors work via different mechanisms of action but each inhibited estrogen dependent cell proliferation (Figure 2.4 A) indicating the effects of picomolar estradiol are mediated through ERα.

To more directly assess the role of ERα in estrogen-dependent cell proliferation induced by pM E_2, we performed an siRNA knockdown of ERα in MCF-7 cells. Because our data suggest a tiny fraction of ERα bound with E_2 is sufficient to stimulate cell proliferation and significant protein remains even after RNAi knockdown, we used pre-treatment with ICI 182,780/Fulvestrant to degrade the ERα already present and subsequently inhibited the synthesis of new ERα with siRNA. ICI alone reduced, but did not eliminate estrogen-induced cell proliferation over the course of the experiment. However, the combination of ICI pre-treatment with siRNA knockdown completely blocked cell proliferation induced by pM E_2 (Figrue 2.4B). The non-coding (NC) control siRNA had no effect.

EGF does not potentiate pM E_2 induced proliferation

While estrogens binding to estrogen receptor activate gene transcription, post transcriptional modification of ER facilitates its downstream effects. Maximum activation of ER requires by phosphorylation of the receptor via ERK 1/2 phosphorylation at Ser-118 and Ser-167[39]. To test whether the EGF pathway could potentiate the effects of low concentrations of E_2, MCF7 cells were treated with levels of E_2 below which maximal cell proliferation was observed given our dose response curves

The results (Figure 2.5) show that addition of 100 ng/ml EGF to the medium did not potentiate the effect of low concentrations of E_2. With and without EGF the cells achieved the same level of proliferation. Thus activation of the ERK pathway by addition of EGF does not further stimulate proliferation induced by pM E_2. These data indicate that, acting via ERα, pM E_2 is sufficient to induce maximal proliferation of ERα positive cancer cells

Activation of the ERK pathway moderately contributes to E_2 induced proliferation

ER has many effects outside the nucleus including activation of the ERK pathway[12] . E_2 binding to ER can cause ERK to be phosphorylated and activated, which in turn can reciprocally maximally activate the receptor. To assess the contribution of ERK activation to proliferation induced by pM E_2, we used the ERK1/ 2 inhibitor, U0126[40]. Pretreating cells with U0126 before the addition of low and high E_2 demonstrated that the major pathway of proliferation is not via the ERK 1/2. Interestingly, the ERK pathway contributed to approximately 30% of the growth induced by pM estrogen (Figure 2.6 A and B). Western blot demonstrated U0126's ability to inhibit phosphorylation of the p44-42 MAPK. The data demonstrate that the ERK pathway activity contributes to low E_2 induced proliferation but is not the major pathway of E_2-ER proliferation.

GPR 30 inhibits pM E_2 induced proliferation

GPR 30 also known as G-Protein Estrogen Receptor (GPER) is a G-Protein Coupled Receptor (GPCR) that that binds estrogen and is localized to the membrane of the endoplasmic reticulum[41-45]. However, its role in estrogen dependent growth has been controversial with some studies indicating that it is an important pathway for estrogen dependent growth while other studies indicate it has minimal importance for estrogen induced proliferation. To assess the role of GPR30 in pM E_2 induced proliferation, we used a known synthetic agonist of GPR30, G1. In the absence of E_2, G1 did not stimulate proliferation of MCF-7 cells. Surprisingly, while G1 is an agonist of GPR30 our demonstrated that it robustly inhibited proliferation induced by 10 pM E_2 (Figure 2.7).

2.5 Discussion

Recent advances in the measurement of estrogen levels from patient samples have revealed that the concentrations of estrogens necessary to increase the risk of breast cancer in postmenopausal women are in the picomolar range[16-18, 20-22]. However, it was unclear whether these levels of E_2 could stimulate the proliferation of breast cancer cells *in vitro*.

Here we describe a model for estrogen dependent growth of breast and ovarian cancer cancer cells in which cell proliferation is entirely dependent upon the addition of E_2 to the growth medium. This cell culture model recapitulates the observation that in mouse xenograft models MCF-7 breast cancer cells fail to proliferate without the addition of estrogen[23, 24]. These assays reveal that picomolar concentrations of estrogen maximally stimulate the proliferation of breast cancer cells in 2D cell culture. This data correlates with human subjects data regarding the level of free E_2 in serum of postmenopausal women. Similar results were obtained in 3D clonogenic assays of estrogen dependent growth (soft-agar assays).

Using the specific ERα antagonists ICI 182 780 and TPSF along with siRNA knockdown, we demonstrate that ERα mediates cell proliferation induced by picomolar E_2. MCF-7 cells treated with the antagonists ICI and TPSF failed to grow. siRNA knockdown was achieved without the control siRNA compromising the ability of the cells to proliferate. Consistent with a role for ERα, we found that cells which contained higher levels of ERα required lower concentrations of E_2 to maximally stimulate proliferation.

We also assessed the contribution of pathways outside of the classical mechanism of ER activation, specifically the extra-nuclear ERK pathway. Our results indicate that activation of the ERK1/2 pathway is a contributor to the proliferation of breast cancer cells but is not the major

pathway by which E_2 functions. This would correlate with previous data demonstrating that an estrogen dendrimer conjugate (EDC), only capable of activating the extra-nuclear effects of ERα, was minimally effective in stimulating cell proliferation[46].

Another alternate pathway of E_2 action that has been extremely controversial has been GPR 30. GPR 30 was originally reported as causing breast cancer cells to grow. However, recent literature has revealed that the binding affinity of GPR30 for E_2 is extremely high and that it appears to inhibit proliferation in ERα positive breast cancer cells when activated[43]. Here we show that activation of GPR30 through a specific ligand, G1, does not stimulate proliferation of ER positive breast cancer cells, actually inhibits their growth similar to recent articles and, therefore, could not be the pathway responsible for low E_2 induced proliferation.

Our data indicates that E_2 and ER function far below the K_d of the receptor for E_2. The data results suggest that stimulating cell proliferation requires a threshold number of ERα molecules that must contain bound E_2. How this threshold is reached differs for each cell type and is a function of the intracellular level of ERα and the concentration of E_2. Although ERα binds to at least 10,000 sites in the human genome[47], our data suggests that far less than 10,000 molecules of E_2-ERα per cell is sufficient to robustly induce cell proliferation.

These results highlight the biological importance of low circulating levels of free E_2 in the growth and proliferation of breast cancer cells and provide a system for studying this in cell culture.

2.6 Figures

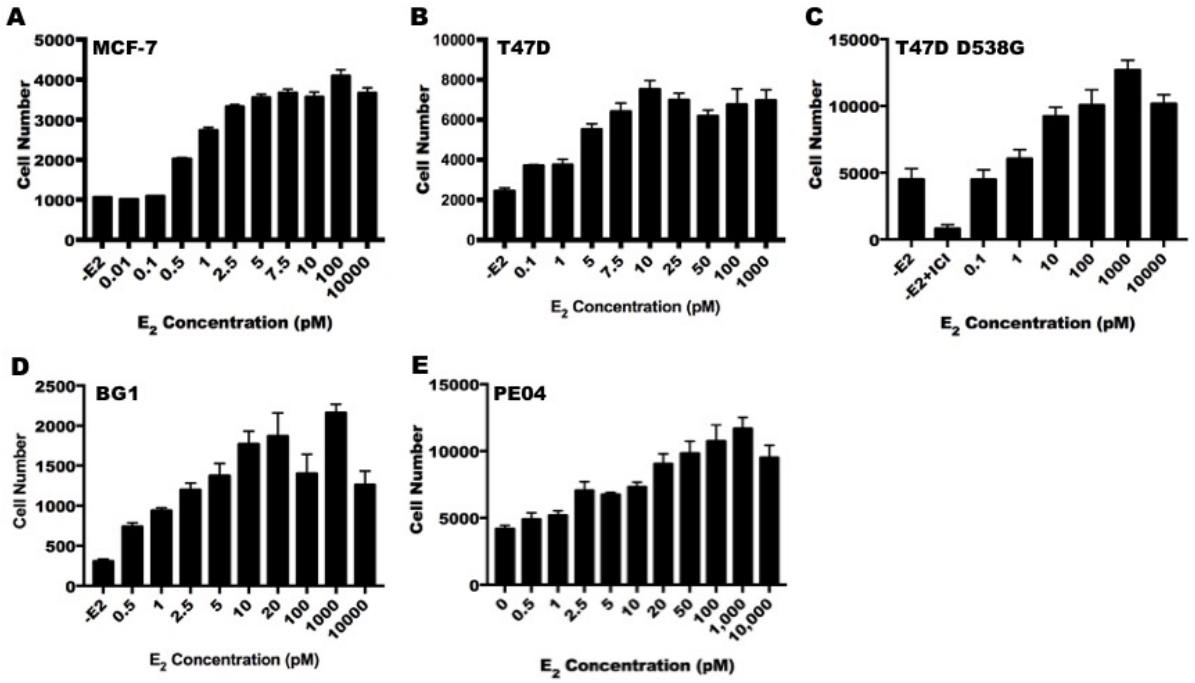

Figure 2.1. Picomolar concentrations of 17β-estradiol elicit near maximal proliferation of breast and ovarian cancer cells. Dose response curves were performed on (A) MCF-7 (B) T47-D and (C)T47D D538G and (D) BG-1 cells. 1,000 MCF7 cells were plated per well and treated with the indicated concentrations of E_2 for: 3 days for MCF7 cells, 4 days for T47D, and 7 days for BG1 cells. Then proliferation was analyzed using MTS reagent. Plating cell number was 1,000 for MCF7, 2,000 for T47D, 2000 for T47D D538G, 200 for BG1 and 500 for PE04 cells. Data points represent mean of 8 experiments ± SEM.

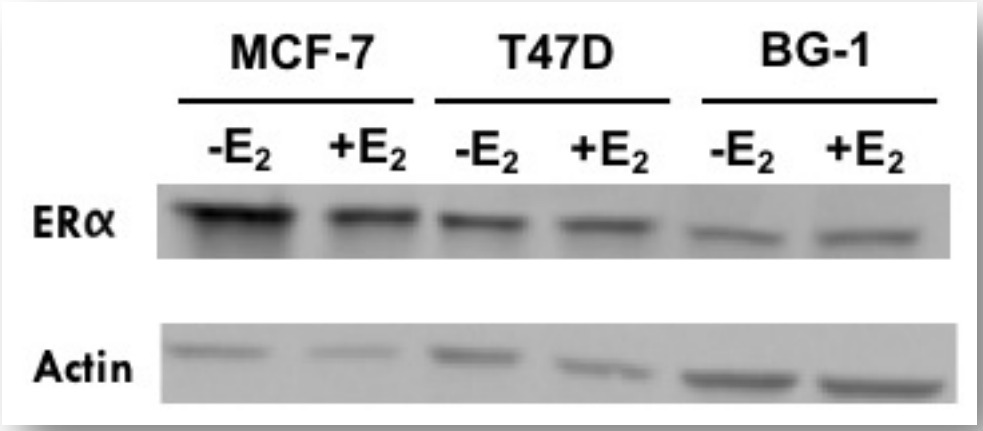

Figure 2.2. Western blot of ERα protein levels. Cells were plated and maintained for 4 days in CD-FBS and plated into 10% CD-CS. 12 hours after plating cells were treated with 10 nM E_2 for 12 hours and whole cell extract was collected. 20 μg of lysate was resolved on a 10% PAGE and probed with anti-ERα and anti-actin antibody.

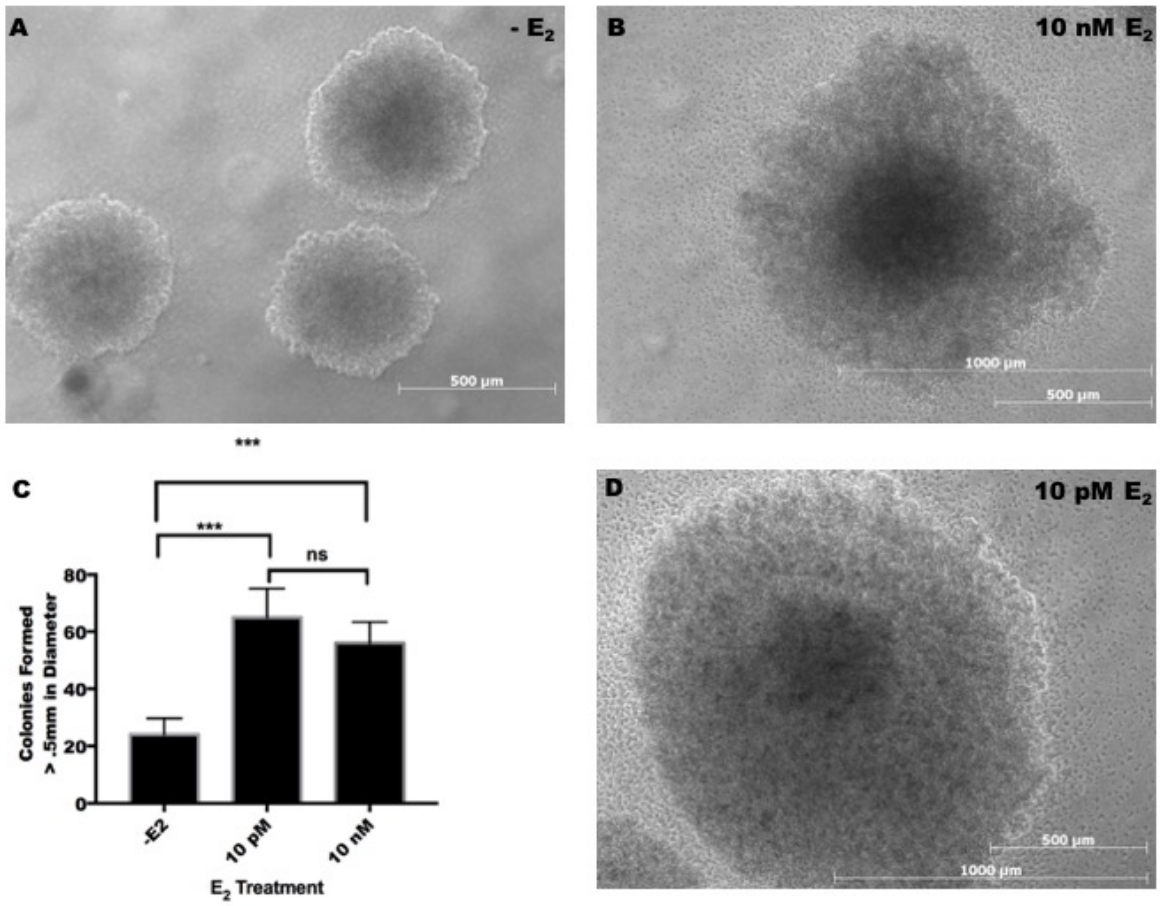

Figure 2.3. 3-dimensional proliferation assay reveals tumorigenic potential of low E_2. Colony formation assays in soft agar assess anchorage-independent growth and clonal outgrowth properties of tumors. MCF-7 cells were treated with either 10 pM or 10 nM E_2 for a period of 21 days. The results of the experiment showed no statistically significant difference in colony forming ability between the Low (10 pM E_2) and High (10 nM E_2) groups. However, the low and high groups showed statistically significant increases in colony formation over the (-)E_2 group (*** represents $p<0.002$).

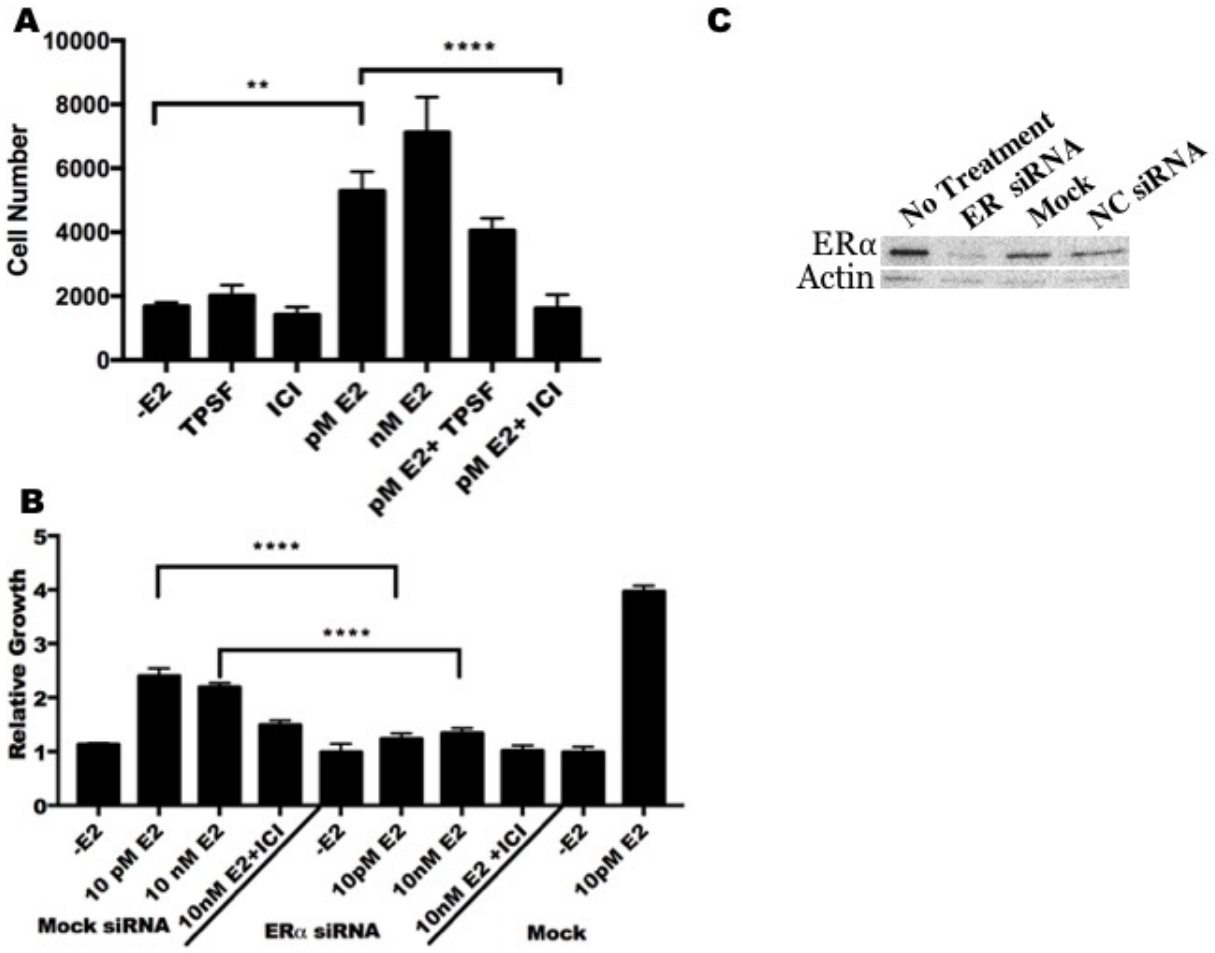

Figure 2.4. Inhibition of ERα abrogates proliferation induced by pM E_2. (A) Cells were maintained in the ERα specific pharmacologic antagonists ICI 182,780 and TPSF for 3 days, with or without E2, and cell number assayed using MTS. (B) 5nM ICI 182,780 was pre-incubated with cells for 12 hours to degrade endogenous ER before siRNA knockdown of ERα was initiated. Cells were transfected for 16 hours before being removed and re-plated in 96 well plates for growth assays. Cells were treated with E_2 after 12 hours of recovery and analyzed using MTS reagent after 3days. (C) Western blot of ER alpha and Actin after siRNA knockdown Results are ± SEM of 3 independent replicates. (** represents $p<0.03$, **** represents $p<0.001$)

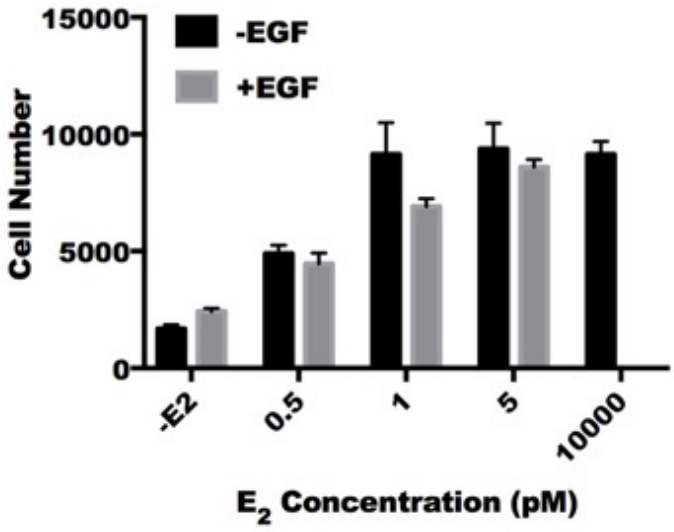

Figure 2.5. EGF does not potentiate proliferation induced by pM E_2. To study the effect of ERK kinase activation, MCF7 cells were treated with 100ng/mL EGF. Cells were incubated with EGF or without EGF and varying concentrations of E_2. Cells were assayed using MTS reagent 3 days later. Results are mean ± SEM of 3 independent replicates.

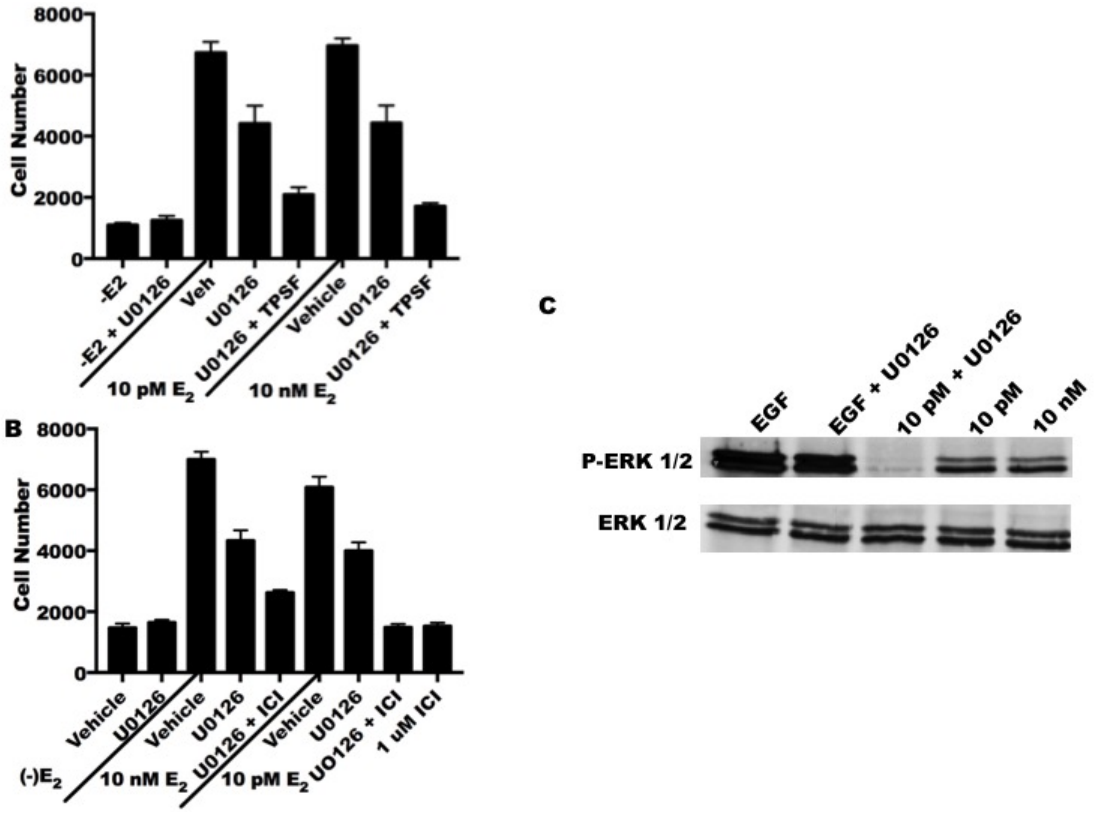

Figure 2.6. The MEK 1/2 U0126 inhibitor partially blocks E_2 Induced Proliferation. MCF-7 cells were pretreated with 10μM U0126 for 2 hours before treatment with E_2 or one of 2 ER specific antagonist A.) TPSF B.) ICI 182,780/ Fulvestrant. Cells were assayed after 3 days with MTS reagent. Results are displayed as mean ±SEM of 3 independent replicates. (C) 20ug lysate were collected for western blot analysis and probed with anti-ERK antibody or anti-phosphoERK antibody

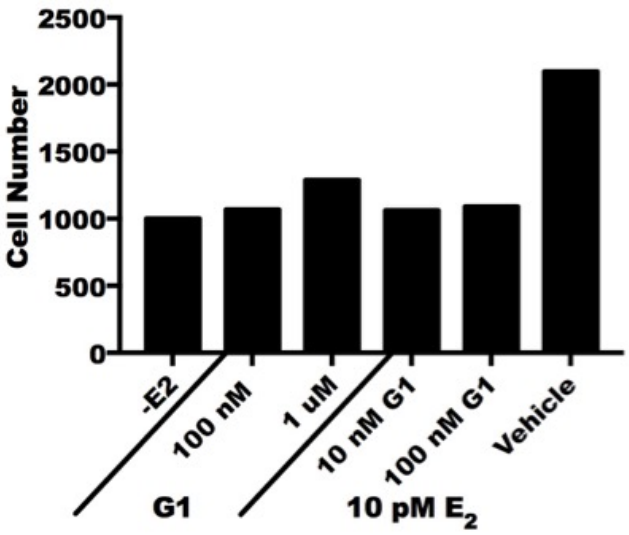

Figure 2.7. GPR30 Agonist G1 Inhibits E_2 Dependent Proliferation. MCF-7 cells were treated either with vehicle, G1 agonist alone or G1 in combination with 10 pM E2. Cell number was determined using MTS reagent after 36 hours of treatment. Results are displayed as mean ±SEM of 3 independent replicates.

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Chapter Three

Picomolar Concentrations of E_2 Regulate Breast Cancer Gene Expression

3.1 Abstract

The circulating levels of 17β-estradiol (E_2) normally found in postmenopausal women are in the low picomolar range as gathered from human sampling. However, it was unclear whether these extremely low E_2 concentrations can stimulate gene transcription. Here we demonstrate that concentrations of E_2 as low as 5 pM can stimulate gene transcription. Comparing the effect of picomolar and nanomolar concentrations of E_2, we find several well-studied E_2-estrogen receptor α (ERα)-regulated genes including pS2 and progesterone receptor (PGR) are induced by pM E_2. We also find that pM E_2 reduces expression of IL1R1, which is normally downregulated by E_2-ERα. However, expression of the E_2-ERα-induced protoconcogene c-fos was not demonstrated at picomolar concentrations of E_2. We also noted that 10 pM E_2 was sufficient to induce the E_2-ERα cell cycle regulator, E2F1, at the protein level. Finally, we demonstrate that picomolar concentrations of E_2 were as effective as nanomolar E_2 in increasing recruitment of ERα to the GREB1 and pS2 promoters. This data demonstrates that picomolar concentrations of E_2 able to ligand only a very small fraction of intracellular ERα pool are sufficient to regulate expression of several estrogen-responsive genes. Since pM E_2 induces robust proliferation of ERα positive cancer cells, differential regulation of E2F1 and cFos suggests that the ability to elicit gene regulation at post-menopausal physiologic concentrations. Therefore, pM E_2 represents a novel

way to identify genes likely to play a role in estrogen-stimulated proliferation of cancer cells and exclude genes unlikely to play a role.

3.2 Introduction

Estrogens acting via the steroid nuclear receptor, estrogen receptor α (ERα), are extremely important in the growth and development of breast cancer[1]. The importance of ER is evidenced by the fact that mainstay adjuvant therapies target estrogen-ERα via a variety of mechanisms. The first method is to block E_2 binding to ER. This is accomplished by drugs such as tamoxifen, raloxifene, and ICI 182780/Faslodex/fulvestrant that compete with estrogens for binding to ERα[2, 3]. The second is to starve cells of E_2 by inhibiting aromatase, the enzyme responsible for the conversion of testosterone to 17β-estradiol [4]. Through either mechanism, the end goal is blocking formation of the E_2-ERα complex which has proven critical for treating breast cancer. It also highlights the importance of the hormone-steroid receptor complex in driving proliferation of breast cancer cells.

Classically the E_2-ERα acts via direct regulation of gene transcription. E_2 and other estrogens produced by the ovary and the adrenal gland bind to estrogen receptors localized in the nucleus. Binding of estrogens to the Ligand Binding Domain (LBD) of estrogen receptors induces a conformational change in the receptor. The conformational change allows for homo-dimerization of the receptor. The receptor dimer can then be recruited to DNA either at palindromic Estrogen Response Elements (ERE) or it can be tethered to DNA through binding to other transcription factors that bind DNA[1, 5-7]. The Activation Function 1 and 2 (AF1 and AF2) domains of the receptor facilitate recruitment of co-activator proteins that aide in assembly of the transcription complex[8, 9].

Both pre and postmenopausal women develop ERα positive breast cancer. However, the majority of women who develop $ER\alpha^+$ breast cancer are post-menopausal[10]. At the point a woman reaches menopause the levels of estrogenic steroid hormone produced are at their nadir[11-13]. A number of recent studies utilizing newer, more accurate techniques have demonstrated that concentrations of circulating E_2 necessary to increase the risk of breast cancer lie in the low picomolar range[14-17].

Chapter 2 demonstrated that picomolar concentrations of E_2 could illicit *near maximal* proliferation of breast cancer cells. The data showed that these miniscule levels of estrogen did not exert their major function via the extranuclear pathway. This implies that the major axis by which picomolar levels of estrogen exert their effect is via classical ERα action in the nucleus. However, it has been suggested that these E_2 concentrations are too low to induce E_2-ERα dependent transcription. The data also imply that a threshold of ER saturation must be achieved in order to elicit proliferation of ERα positive breast cancer cells. To fully assess and understand the effect of low E_2 concentrations on gene transcription, we undertook a series of studies that looked not only at concentration dependent gene transcription but also sought to identify differentially regulated genes within the E_2 -ERα target universe important for breast cancer cell proliferation.

3.3 Materials and Methods

Cell culture

Cells were maintained in MEM supplemented with 10 mM HEPES and either 5% (MCF-7 and BG-1) or 10% (T47D) FBS. 4 days prior to plating cells were cultured in MEM supplemented with 5% (MCF-7 and BG-1) or 10% charcoal-dextran treated CD-FBS.

RNA isolation

Cells were grown for 4 days in 5% CD-FBS (MCF-7 and BG-1) or 10% CD-FBS (T47D). Cells were trypsinized and transferred to a 6-well plate at 60% confluency in 10% CD-CS. Cells were allowed to adhere for 12 hours and then medium containing the indicated treatment conditions was added to the cells. Cells were lysed and RNA was purified using the mini-RNAeasy kit (Qiagen). RNA was quantified using spectrophotometer and a minimum A260/A280 ratio of 1.8 was used to as a cutoff for RNA.

cDNA Synthesis and qRT-PCR

1 μg of isolated RNA was reverse transcribed to cDNA using a cDNA synthesis kit (NEB). 50 ng of reverse transcribed cDNA was loaded in each reaction in a 96-well plate, along with Sybr green (Roche) and 150 nM of gene-specific primers (Table 1). Reactions were run as a 2 step PCR reaction on an Applied Biosystems Step One Plus Real Time PCR system for 40 cycles at 90°C for 15min; the resulting C(t) values were compared using the ΔΔC(t) method[18].

Western blot

Cells were cultured in 5% CD-FBS (MCF7 and BG-1) or 10% CD-FBS (T47D) for 4 days prior to being plated at 300,000 cells/well in 6-well plates in medium containing 10%CD-CS. Whole cell extracts were prepared after 24 h of treatment using 1× radioimmune precipitation assay buffer (Millipore) containing complete mini protease inhibitor mixture (Roche). 30 μg of protein per lane was analyzed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). E2F1 protein was detected using E2F1 antibody 3742 (Cell Signaling), internal control α-tubulin was detected using monoclonal antibody T1699 (Sigma), and β-actin was detected using antibody A1978 (Sigma).

ChIP

MCF-7 cells were maintained in 5% CD-FBS for 3 days and treated with 10 nM E2, 10 pM E2, or Vehicle for 45 min. Cells were cross-linked with 1% formaldehyde for 15 min. Cell extracts were digested for 10 min with 50 units of micrococcal nuclease (NEB) at 37 °C and further sonicated to yield sheared DNA fragments with an average length of 200–1000 base pairs. The sonicated samples were pelleted by centrifugation, and the supernatant was diluted 5-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and protease inhibitor mixture). 50 µl of diluted supernatant was reserved as input (10%) for each treatment. The samples were precleared with 50 µl of protein A-Sepharose™ 4 Fast Flow (GE Healthcare) in ChIP dilution buffer (1:1) preblocked with 200 µg/ml sheared herring sperm DNA and 500 µg/ml BSA. The samples were then divided, and the remaining proteins were incubated with either 2 µg of anti-ERα (C19), 2 µg of anti-RNA polymerase II (clone CTD4H8, Millipore), or control mouse IgG overnight at 4 °C. The antibody-protein-DNA complex was precipitated by incubating with 50 µl of Protein A-Sepharose^{TM} beads for 2 h at 4 °C. The protein-DNA complex was eluted from the beads with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed, and DNA was eluted from the protein-DNA complexes by adding 200 mM NaCl and incubating overnight at 65° C. Protein was digested by incubation at 45 °C for 2 h with Proteinase K. DNA was recovered and purified. Quantitative PCR was performed to determine the change in ERα and RNA polymerase II occupancy at various sites of ERα binding. The double negative controls were nonspecific antibody (normal mouse IgG) and primers coding for intergenic regions that do not interact with ERα. Thermal cycling conditions were 95 °C for 10 min followed by 50 cycles of 25 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C.

Statistical analysis

All statistical analysis was performed using two-way ANOVA with Bonferroni correction.

3.4 Results

Dose-response study of E_2 induced transcription

Since it is not fully understood whether E_2 concentrations far below the K_d of estrogen receptor can stimulate the transcription of genes it was important to ascertain the concentrations of E_2 that could induce transcription. As a test gene we chose the classic readout of E_2-ERα mediated gene expression, pS2. pS2 is regulated directly by E2-ERα through direct DNA binding at Estrogen Response Elements (EREs). The data (Figure 3.1) demonstrate that at E_2 concentrations in the physiologic postmenopausal range induce transcription of the well-studied E_2-ERα regulated gene, pS2. (15). E_2 stimulated detectable levels of pS2 mRNA at 5 pM and 10 pM E_2. However, E_2 failed to induce pS2 mRNA at 1 pM E_2. Interestingly, concentrations of E_2 in the femtomolar range that we show stimulate half maximal proliferation of MCF-7 cells (Chapter 2) did not have any effect on pS2 gene transcription.

Maximal induction of pS2 mRNA occurred at 100 pM E_2 and not 1000 pM E2. While this was surprising it does correlate with previous data regarding peak levels of E_2-ERα induced gene transcription in artificial systems[19, 20]. In those systems, a stably transfected luciferase reporter with 3 Estrogen Response Elements reached maximal luciferase activity at 50 pM E_2. This would suggest that hormone concentration and transcription are not linearly related at all levels of E_2. The data clearly demonstrate that physiologic concentrations of 17β-estradiol can stimulate gene transcription, but gene expression does not reach the levels seen at higher E_2

concentrations. This also indicates that E_2 can exert its classical effects far below the measured K_d for binding of E_2 to ERα [21, 22].

Time course of E_2-ERα induction of MCF-7 gene expression

Given that only a small fraction of the total estrogen receptor population is ligand bound at picomolar concentrations of E_2 and that this fractional population is capable of stimulating cell proliferation and gene expression, it would be reasonable to extrapolate that only a fraction of the ERα transcriptome is being transcribed at low concentrations of E_2[23] . The transcriptome would include putative ERα genes important in proliferation. Therefore, using qPCR we investigated differential regulation of genes at low E_2 (10 pM E_2) and high E_2 (10 nM E_2).

The data (Figure 3.2 A-D) show a sample of genes tested. Figure 3.1B reveals that pM E_2 robustly stimulated the transcription of the pS2/TFF1 gene to a fold induction not previously reported in the literature. However, since the biological function of pS2 is not well defined, we wished to assess E_2-ERα-regulated genes with known biological functions. Therefore, we looked at progesterone receptor (PGR), cMyc, Cyclin E2, cFos, Cyclin D1, and IL1R1 all of which are reported ERα targets (Figure 3.2 A-F)[24-28]. PGR (Figure 3.2A) mRNA was induced at both low and high E_2; with the fold induction al 10 pM E_2 approximately one half the maximum seen at 10 nM E_2. cMyc another putative ERα target demonstrated close to 100-fold induction with nM concentrations of E2 but was not nearly as well induced by picomolar concentrations (Fig 2B). However, c-fos, a gene proposed as a critical mediator of E_2-ERα induced proliferation was not measurably induced by 10 pM E_2 but remained responsive to E_2-ERα at 10 nM E_2 (Fig 1C)[29]. According to this data, since 10 pM E_2 induced robust proliferation of MCF-7 cells induction of c-fos does not play a central role in E_2-ERα stimulated cell proliferation

E_2-ERα complex can both increase the expression of certain genes and decrease the expression of other genes. ER downregulates genes through tethering mechanisms and not via direct interaction with DNA. To understand whether picomolar E_2 could downregulate a known ERα target gene we chose IL1R1, which is known to be downregulated at nanomolar concentrations of E_2[30]. E_2-ERα diminished transcription IL1R1 mRNA at both high and low concentrations of estrogen (Fig. 3.1D).

The qRT-PCR data demonstrates that the ligand-receptor complex at different concentrations of estrogen differentially regulates several E_2-ERα target genes. This is true for target genes regulated by different mechanisms of action. Upregulated ERα target genes are known to act via direct DNA binding and tethering while downregulated genes are proposed to work solely via tethering mechanisms.

This data shows differential regulation of ERα target genes at different E_2 concentrations. The data also show that for a 1000-fold difference in E_2 concentration there is only, in general, an ~50% difference in transcriptional activity demonstrating a dramatic distinction between ERα saturation and transactivation by ERα.

E2F1 protein expression is stimulated by low E_2

To test whether increases in transcription of genes implicated in E_2-ERα dependent proliferation of MCF-7 cells lead to increases in the level of protein expression, we carried out western blot analysis of a putative E_2-ERα-regulated cell cycle progression gene, E2F1. The E_2-ERα-induced E2F1 gene has gained prominence as a critical cell cycle factor implicated in E_2-ERα induced cell proliferation[31].

Western blot of E2F1 protein at low E_2 (10 pM) and high E_2 (10 nM) provided strikingly similar results. The E2F1 protein was expressed at 8 and 12 hours post E_2 in both samples. Densitometry revealed no statistically significant difference between the levels of protein at 12 hours of induction (Figure 3.3B). The levels of E2F1 protein demonstrate that picomolar E_2 can stimulate the production of E_2-ERα-regulated proteins. A 1000-fold difference in E_2 concentration, resulting in increased saturation of ERα with E_2, had no effect on the level of a key E_2-ERα induced protein.

Increasing the E_2 concentration 1,000-fold does not increase recruitment of E_2-ERα to promoters

qRT-PCR data provided information on the relative levels of target E_2-ERα mRNAs. We wished to explore the influence of E_2 concentration on ERα recruitment to promoters of E_2-ERα-regulated genes containing known EREs. Therefore, we conducted Chromatin Immunoprecipitation (ChIP) assays at 10 pM E2 and 10 nM E2.

pS2 and GREB1 are two well studied estrogen regulated genes[32-36]. At 10 pM E_2 and 10 nM E_2 ERα is recruited equally to the promoter of both genes (Figure 3.4). The increase in recruitment over the (-) E_2 sample is statistically significant ($p<0.001$) for both high and low E_2. Interestingly, for both genes RNA polymerase II recruitment, was ~50% lower at 10 pM E_2 than 10 nM E_2-ERα. This correlates with the qRT-PCR data showing that induction of pS2 and GREB1 mRNAs at 10 pM E_2 was approximately half of the induction seen at 10 nM E_2.

3.5 Discussion

In Chapter 2 we demonstrated that picomolar concentrations of E_2 induce near-maximal proliferation of breast cancer cells and the major estrogen receptor axis through which proliferation is induced is via the classical genomic mechanism of ERα action. However, it was still poorly understood whether these minute concentrations of E_2-ERα could lead to quantifiable induction of gene transcription. Here we provide direct evidence that picomolar concentrations induce transcription of known ERα target genes such as pS2 in a physiologically relevant of E_2 concentration. We also show that pM concentrations of E_2 differentially regulate ERα target genes compared to high nanomolar concentrations. While genes such as pS2 and PGR are extremely responsive to a very low concentration of E_2, we found that that c-fos, a putative oncogene, was not induced at low concentrations of E_2 but was robustly induced at nanomolar concentrations of E_2[29].

Picomolar concentrations of E_2 were also able to induce the protein E2F1, a known cycle regulator which has been proposed to play an important role in E_2-ERα-stimulated proliferation of cancer cells[31]. E2F1 showed a strikingly similar level and time course of induction at picomolar and nanomolar concentrations of E_2 substantiated by densitometry.

ChIP studies on the GREB1 and pS2 promoters demonstrate that at low and high concentrations of E_2, ERα is recruited equally to both promoters. However, the increase in recruitment of RNA polymerase II at low pM E_2 was only half of that seen with high nM E_2. RNA polymerase II recruitment was better correlated with the level of induced mRNA than ERα recruitment. These data suggest that because ER is readily recruited to the promoter of two genes it regulates directly via DNA interactions the rate limiting step in gene transcription may not be

the initial recruitment of E_2-ERα to promoters. Instead, recruitment of co-regulator proteins and assembly of a transcription complex leading to RNA polymerase II may represent the rate-limiting step.

Most of our studies take place over a thousand-fold range of E_2 concentration, 10 pM and 10 nM. Since the K_d for binding of E_2 to ERα is ~0.5 nM, 10 nM E_2 will saturate all the ERα with hormone, while only a very small percentage of the ERα will be saturated with E_2 at 10 pM[21, 22]. However, on some, but not all, genes this large difference in the percentage of ERα containing bound E_2 only results in an approximately 2-fold change in the level of induced mRNA. This suggests that the percentage of ERα saturated with E_2 has only a limited impact on biological outcomes at many genes in breast cancer cells.

While nearly all studies use saturating concentrations of E_2 to analyze ERα recruitment to target genes, we show that physiologically relevant concentrations of E_2 effectively recruit ERα to at least some estrogen regulated promoter regions. Our data complements and extends current epidemiologic data suggesting that picomolar concentrations of serum 17β-estradiol are correlated with a highly increased risk of breast cancer [14-17, 37]. However, this also has major implications for what remains unknown about breast cancer. The eminent cancer biologist, Dr. Robert Weinberg, in "*The Biology of Cancer"* states that, "breast cancer…has been the subject of extensive research, yet we still have only a vague understanding at the molecular level of how growth of these tumors is controlled"[38]. While Dr. Weinberg may overstate what is *not* known about breast cancer, he does point out that we have identified a large number of cell cycle regulators thought to be critical for E_2 induced proliferation. Using our observation that some genes fail to exhibit regulation by low concentrations of E_2, we can use expression-profiling to narrow down the ER target gene universe important for the proliferation of breast cancer cells

(Figure 3.5). This would allow one to exclude genes, such as c-fos that require nanomolar E_2 concentrations for regulation and include genes regulated both at picomolar and nanomolar concentrations of E_2.

Furthermore, the data presented here is relevant to an important recent study indicating that that almost 15% of the genome is regulated by ERα[39]. While the conclusion that a major portion of the genome may be regulated by ER is probably correct the high 100 nM concentration of E_2 used in that study suggests that there is a gradient of response to E_2 as its concentration increases. Some genes, such as c-fos will only respond to E_2 *in vivo* in cells containing high levels of ERα and during phases of the menstrual cycle or in pregnancy when levels of circulating levels E_2 are high[12, 13]. At 10 pM E_2 only a few hundred molecules of ERα will contain bound E_2. At this concentration of E_2, which is in the range seen in postmenopausal women, it is likely that only a fraction of the potential E_2-ERα-regulated transcriptome responds to estrogen.

Further understanding of the genes and mechanisms of regulation of E2 may allow us to explain differential actions of E2 over the course the hormone cycle. It is also possible that this may lead to answers regarding transcription dynamics that have recently raised[40]. Eventually characterizing the highly estrogen responsive fraction of the E_2-ERα-regulated transcriptome may be the preferred target for evaluation of next-generation ERα inhibitors.

3.6 Figures

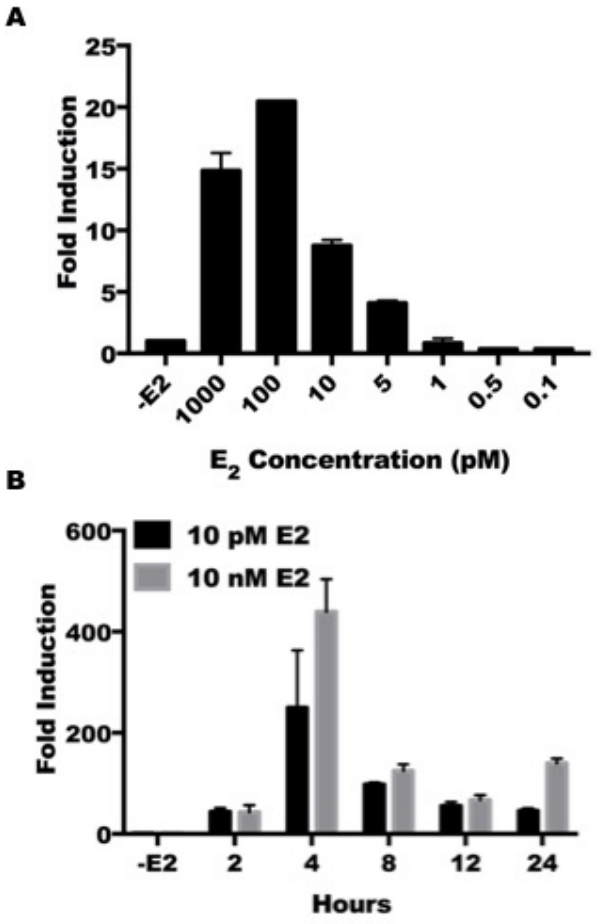

Figure 3.1. Dose Response and Time Course Study of E_2-ER*α* induction of pS2 mRNA. MCF-7 cells were maintained in 5% CD-FBS for 4 days and re-plated in 10% CD-CS. Cells were treated with indicated concentrations of E_2 for 4 hours, RNA was collected and reverse transcribed to cDNA and quantitated using the qPCR ΔΔ C(t) method. Data represent mean fold induction over (-) E2/Vehicle ±SEM. (A) pS2 dose response to varying doses of estrogen. (B) Time course of pS2 mRNA induction

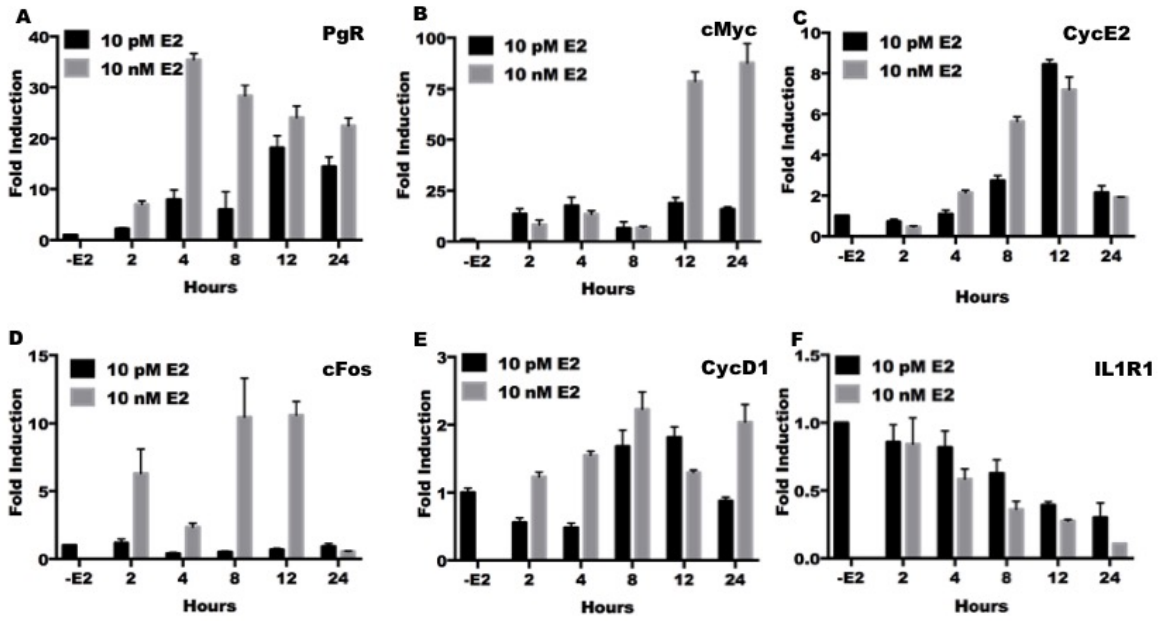

Figure 3.2. ERα Target Genes are Differentially Regulated by Low and High E_2. MCF-7 cells were maintained in 5% CD-FBS for 4 days and re-plated in 10% CD-CS. Cells were treated with high (10 nM) E_2 or low (10 pM) E_2 for for 2, 4, 8, 12 and 24 hours. RNA was collected and reverse transcribed to cDNA and quantified using the qPCR ΔΔ C(t) method. Data represent mean fold induction over (-) E2/Vehicle ±SEM. (A) PgR (B) cMyc (C) CycE2 (D) cFos (E) CycD1 (F) IL1R1.

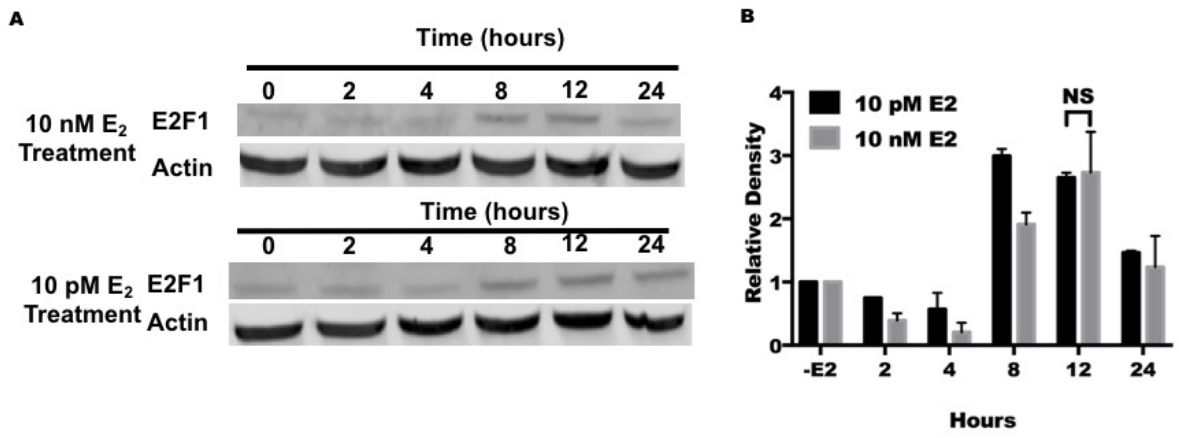

Figure 3.3. A low concentration of E_2 induces E2F1 protein. After 4 days in 5% CD-FBS, MCF-7 cells were plated in 10%CD-CS. Treatment medium containing 10 pM E_2 or 10 nM E_2 was added to the cells. Lysate was collected at 2, 4, 8, 12, 24 hours using RIPA buffer. 40 ug of total protein was run for each condition and probed using anti-E2F1 antibody or anti-actin.

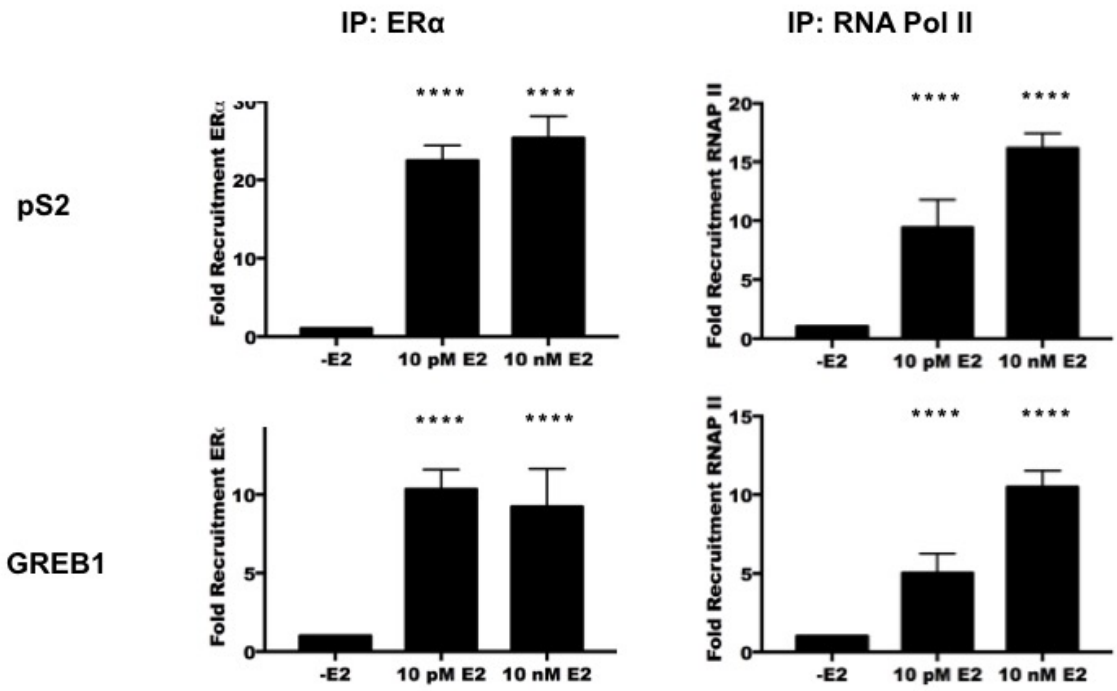

Figure 3.4. Low and high E_2 concentrations result in equivalent ERα recruitment to EREs but decreased RNAPII recruitment. MCF-7 cells were maintained in 5% CD-FBS for 3 days prior to the experiment. Low E_2 (10 pM E_2) and high E_2 (10 nM E_2) were added to the cells for 45 min. Protein complexes were crosslinked and immunoprecipitated using ERα antibody and RNAPII antibody. Fold enrichment over IgG was plotted. Data represent the mean of 3 experiments ±SEM. Significance was achieved at $p<0.0001$.

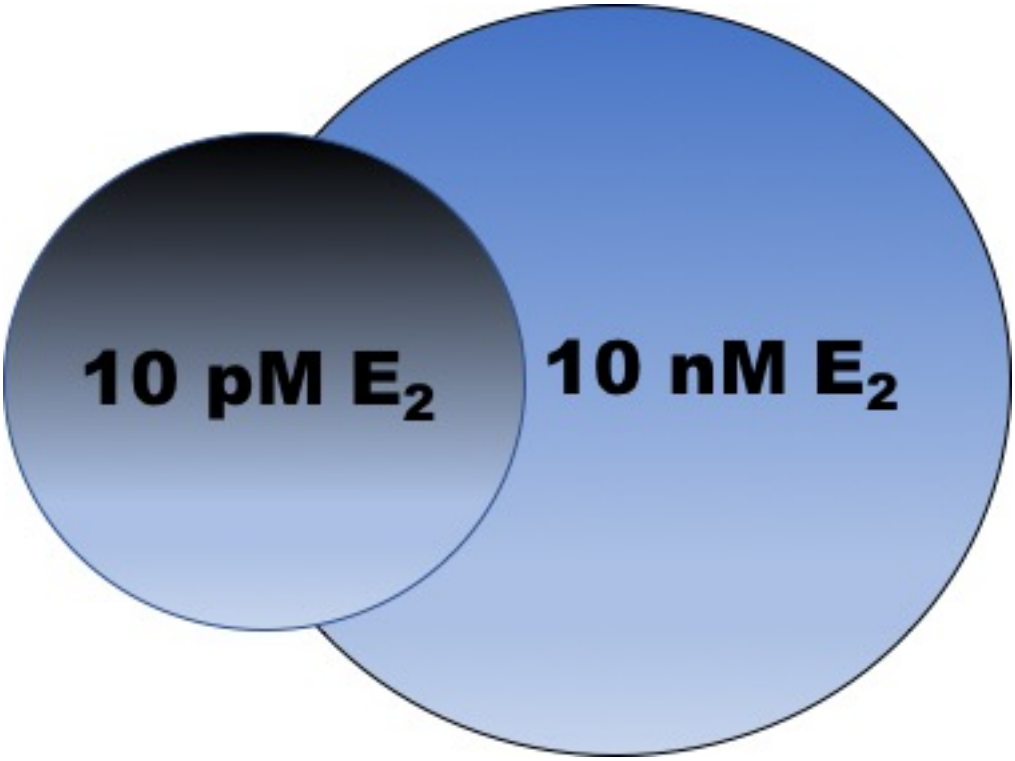

Figure 3.5. Using Low E_2 to catalog the ERα transcriptome. The venn diagram represents how we can use the low E_2 and high E_2 concept to narrow the ERα transcriptome. potential overlap between target universes that could be explored using expression profiling arrays.

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Chapter Four

A New Experimental Model in the Search for Genes E_2-ERα Uses to Stimulate Cell Proliferation

4.1 Abstract

Estrogens acting through Estrogen Receptors (ER) are critical mediators of breast cancer cell growth as evidenced by the effectiveness of tamoxifen and aromatase inhibitors as adjuvant therapies in breast cancer. While ER remains a viable therapeutic target, which genes ERα regulates to make breast cancer cells proliferate has remained an elusive question in the Estrogen Receptor field. The use of cell lines as tools to compare 17β-estradiol (E_2) dependent and independent proliferation has been a mainstay in efforts to identify key genes. However, a major limitation in many systems has been either phenotypes are too subtle to fully characterize, or the cells come from diverse genetic backgrounds making identification of differentially regulated genes challenging. Here we describe a new tool which addresses many of the issues regarding the identification of downstream E2-ERα mediators. We have characterized two cell lines, wild type T47D cells and T47D-KBluc cells. The cell lines are nearly isogenic since the T47D-KBluc cells are derived from wild type T47D cells. The T47D-KBluc cells contain a stably transfected $(ERE)_3$-luciferase reporter. However, unlike the parental cell line the KBluc cells do not proliferate in response to E_2. We demonstrate that the ERα in the KBluc cells is functional on both the artificial reporter gene and on an endogenous gene. We also tested several ERα regulated genes proposed to have critical functions in ERα mediated growth. These genes did not exhibit differential regulation by E_2 in the two cell lines, suggesting they are not responsible for the defect in E_2 induced proliferation. Overall, we characterize a new experimental model that can assist in identifying E_2-ERα regulated genes important in cell proliferation.

4.2 Introduction

The search for ERα regulated genes central to the growth of breast cancers has generated a variety of tools. These tools include cell lines derived from patient tumor samples. In the estrogen receptor field, the predominant cell lines studied are the T47D and MCF-7 cell lines. Each of these cell line is E_2 dependent for proliferation as seen in the data in Chapter 2. However, to take advantage of expression arrays, RNA seq and bioinformatics a number of additional cell lines with unexpected or differential responses to E_2 and ERα have been developed.

Two of the most notable examples developed to tackle these problems have been the MDA-MB-231 ERα+ (231 ERα+) cells and the non-transformed MCF-10A ERIN cells (ERIN). MDA-MB-231 cells are triple a negative cell line (ER-/PR-/HER2-) which resulted in a unique phenotype. When these cells were stably transfected with ERα proliferation is inhibited[1, 2]. The ERIN cells are a derived from immortal but non-tumorigenic MCF-10A cells. The ERIN cells have had ERα stably introduced, but the effects are modest[3].

Both cell lines are successful models of the differential effects of ERα and allowed for the identification of ERα target genes important in proliferation. However, each has major limitations with their ability to identify genes important in E_2 induced cell proliferation. While the MCF-10A ERIN cells grow in response to E_2, proliferation is quite modest and the culture of these cells depends on several supplemented growth factors in addition to E_2 [3]. Therefore, it is difficult to assess the contribution of E_2 to growth as compared to the supplemented growth factors. In a recent paper characterizing the differences in gene regulation between MCF-7 and 231 ERα+ cells, Prof. Benita Katzenellenbogen and coworkers acknowledged the limitations of

the system in recapitulating what was discovered in the 231 ERα+ cells back to a conventionally used cell line such as the MCF-7 cells[4]. This was likely due to large differences in genetic background of the two cell lines. These differences have limited the usefulness of most studies comparing different cell lines.

Here we describe a potential new experimental model for use in the search for ERα regulated genes. We identified two nearly isogenic cell lines that exhibit a differential response to estrogens. The T47D WT cells were shown in chapter 2 to be dependent on estrogens for growth; with their proliferation stimulated by extremely low picomolar concentrations of E_2. In contrast, proliferation of a sub-clone of the wild type cells, T47D-KBluc (KBluc) cells robustly proliferates without estrogen and is completely unresponsive to additional E_2 under the same growth conditions. We characterize these cells and demonstrate the deficiency is not due to lack of biologically active ERα. Due to their nearly isogenic background and on/off phenotype these cell lines represent a useful new experimental model for investigations of genes and pathways important in E_2-ERα stimulated cell proliferation.

4.3 Materials and Methods

Cell Culture

T47D and T47D-KBluc cells were maintained in phenol red containing MEM supplemented with 10 mM HEPES, pH 7.4 and 10% FBS. 4 days prior to plating cells were cultured in phenol red free MEM supplemented with 5% or 10% CD-FBS.

Cell Proliferation Assays

Cells were cultured in medium containing charcoal dextran stripped serum as described above. Cells were trypsinized and resuspended in 10% CD Calf Serum. Cells were plated in a 96-well plate at a density of 1,000 cells/well (MCF-7), 2,000 cells/well (T47D, and T47D-KBluc) or 200 cells/well (BG-1). After 12 hours plating medium was removed and replaced with treatment conditions in 10% CD-CS . After 3-4 days the cells were incubated with Cell Titer 96 Aqueous One Solution (Promega) and analyzed using a spectrophotometric plate reader at 490 nM and 650 nM. A standard curve was always performed to correlate cell number to absorbance.

RNA Isolation

T47D and T47D-KBluc cells were grown for 4 days in 10% CD-FBS. Cells were trypsinized and transferred to a 6-well plate at 60% confluence in 10% CD-CS. Cells were left for 12 hours to adhere and then treatment conditions were added to the cells. Cells were lysed and RNA was purified using mini-RNAeasy kit (Qiagen). RNA was quantified using a spectrophotometer and a minimum A260/A280 ratio of 1.8 was used as a cutoff for RNA.

cDNA Synthesis and qRT-PCR

1 μg of isolated RNA was reverse transcribed to cDNA utilizing a cDNA synthesis kit (NEB). 50 ng of reverse transcribed cDNA was loaded in each reaction in a 96-well plate, along with Sybr green (Roche) and 150 nM primers (Table 1). Reactions were run as a 2 step PCR reaction on an Applied Biosystems Step One Plus Real Time PCR system for 40 cycles at 90° C for 15min. C(t) values were compared using the ΔΔ C(t) method.

Western Blot

Cells were cultured in 10% CD-FBS (T47D and T47D-KBluc) for 4 days prior to being plated at 300,000 cells/well in 6-well plates in medium containing 10%CD-CS. Whole cell extracts were prepared after 24 h of treatment using 1× radioimmune precipitation assay buffer (Millipore) containing complete mini protease inhibitor mixture (Roche). 30 μg of protein per lane was analyzed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). ERα was detected using SC8002 (Santa Cruz Biosceinces) and β-actin was detected using antibody A1978 (Sigma).

4.4 Results

Estrogen Does not Stimulate Proliferation of T47D-KBluc cells

T47D cells are a luminal breast cancer cell line described by Keydar et al in 1979[5]. This cell line has been a prominent cell culture model for ERα positive breast cancer. Therefore, these cells were a natural starting point for development of a stably transfected luciferase based screening system for evaluating the estrogenic potential of environmental compounds[6]. The resulting cell line, T47D-KBluc cells (KBluc), are a sensitive luciferase reporter system maintained without the use of a selection marker.

However, as the results (Figure 4.1) show, proliferation of the stably transfected KBluc cell line is not stimulated by E_2. This contrasts with the data shown in Chapter 2, where the wild type T47D cells proliferate in response to picomolar concentrations of E_2. The KBluc cells grow normally in full serum (10% FBS), and like parental T47D cells do not proliferate in estrogen-free 10% CD-CS used in cell proliferation assays. The KBluc cells neither proliferate nor

undergo apoptosis in response to E_2. Instead, the cells remain quiescent when exposed to estrogens.

Therefore, these two cell lines provide a nearly genetically identical system in which we can study differential responses to estrogen treatment in order to identify genes that are important in E_2-ERα induced stimulated cell proliferation.

T47D KBluc Cells Contain Functional ERα

Since KBluc cells were produced by another lab for a different purpose it is possible that there was inadvertent selection of a dysfunctional reporter. However, the evidence from the development of the KBluc cells[6] and their subsequent use in our laboratory for high throughput screening for ERα inhibitors suggests they contain functional ERα[7]. To confirm that ERα is present western blot analysis of the cells (Figure 4.2C) demonstrated that the KBluc cells contain estrogen receptor and the expression levels are similar to what is seen in their wildtype counterpart.

The cells were stably transfected with a luciferase reporter containing three upstream Estrogen Response Elements (ERE) to drive the transcription of the firefly luciferase reporter (Figure 4.2A). Luciferase reporter data (Figure 4.2B) demonstrates that the $(ERE)_3$-luciferase reporter is functional in these cells and they remain highly responsive to E_2 on a transcriptional and translational level [6-9].

However, an artificial luciferase reporter does not completely recapitulate the cell-based mechanisms important in endogenous gene transcription. To assess whether ERα still functioned on an endogenous gene, qPCR was performed to see whether E_2, acting through ERα, could induce the transcription of a known ERα regulated gene, pS2[9]. The results (Figure 4.2D)

demonstrate that, at nanomolar E_2 concentrations, the induction of the pS2/TFF1 mRNA was nearly equal in the 2 cell lines. This illustrates that the ERα in the KBluc cells is still functional and able to regulate an endogenous gene. pS2 is a classical ERα regulated gene containing a near consensus ERE.

The luciferase and pS2 qPCR data demonstrate that the failure of estrogen to stimulate proliferation of the T47DKBluc cells in not due to loss or dysfunctional ERα. Therefore, this suggests other potential mechanisms, including the disruption of regulation of an endogenous ERα regulated gene, as the cause of the defect in proliferation.

Comparing expression of key E_2-ERα-regulated genes in the T47D and T47D-KBluc cells

Since the KBluc cells contain functional ERα but do not proliferate in response to E_2 they provide a useful tool in comparison to their wild type counterpart for studying ERα regulated genes. Therefore, we decided to explore a small panel of proposed genes to see if their transcription was dysregulated in the KBluc cells.

The results (Figure 4.3A-E) show the qPCR data for several known ERα regulated genes proposed to be key regulators of E_2-ERα signaling. The panel of genes included FOS, ESR2, XBP1, sp-XBP1, and GREB1[10-17]. Interestingly, none of the genes were differentially regulated in the 2 cell lines.

FOS is a proposed ERα target gene and as shown in Chapter 3 it was not induced by picomolar concentrations of E_2 indicating that it may not be a key regulator of estrogen induced cell proliferation[17]. Similarly we observed that there was no significant difference between induction of the FOS gene in the E_2 stimulated cell line and the KBluc cells.

GREB1 is an ERα target gene known to be regulated via an upstream Estrogen Response Element (ERE) and a key chromatin bound regulatory co-factor in ERα induced transcription. It has been shown to be upregulated in breast cancer and an independent prognostic indicator. In figure 4.3C we see that GREB1 is not differentially regulated in the two cell lines. Again this indicates that this is not one of the possibly misregulated factors important for estrogen induced cell proliferation.

Figure 4.3C shows data for ESR2 the gene which encodes the second isoform of estrogen receptor ERβ. ERβ is expressed in many tissues throughout the body but unlike ERα it has been shown to be anti-proliferative in the mammary gland [18-22]. Since ERβ acts as a tumor suppressor it should either be overexpressed or remain similarly regulated in the KBluc cells. The data shows that ESR2 mRNA is neither increased in response to estrogen or differentially regulated in when compared to the wild type cells.

Figures 4.3D and 4.3E are two genes which form part of the IRE1α arm of the Unfolded Protein Response (UPR). The UPR is a critical mediator of translation within cells and has been shown to be directly regulated by Estrogen Receptor α to prepare the cell for subsequent proliferation[8, 23-25]. XBP1 is regulated by ERα as demonstrated via chromosome wide mapping of ER binding sites. Once the UPR is activated, XBP1 is alternatively spliced and eventually a protein product called sp-XBP1 is synthesized. sp-XBP1 is a transcription factor and it regulates a number of UPR-related genes to stimulate production of proteins that allow for correct folding and intracellular transport of proteins. In our system XBP-1 and sp-XBP1 are both upregulated in response to E_2 in both cell lines. This critical pathway remains intact at the mRNA level and does not behave aberrantly in the KBluc cells as compared to the T47D cells.

Here we find that no member of a small panel of genes tested in the T47D KBluc in comparison to their wild type counterpart was dysregulated. Since E_2 does not stimulate proliferation of the KBluc cells, but exhibits normal regulation of these genes, it is possible that these genes are not critical for estrogen-induced proliferation. However, it is also possible that the tested genes are members of ERα regulated critical pathways which have remained unaltered by the process that deprived the KBluc cells of estrogen inducible proliferation. The panel of genes tested is small and represents only a small fraction of the pathways estrogen receptor regulates meaning that there remain a number of ERα target gene or genes which may be misregulated.

4.5 Discussion

Here we describe a novel system consisting of two nearly isogenic cell lines with a differential response to E_2. The KBluc cells, derived from the wild type T47D cells, do not proliferate in response to E_2. This deficiency is not due to a lack of ERα or the ability of ERα to regulate genes. In fact, in the KBluc cells estrogen induces the transcription of the artificial reporter with which they have been stably transfected as well as endogenous ERα regulated genes.

Since one cell line is derived from the other, the cells are nearly isogenic which provides an important advantage over previous experimental models. A key issue in previous work has been whether the considerable difference in genetic background between cell lines being compared, is itself responsible for substantial differences in gene expression (4) . Also, under our conditions the KBluc cells neither proliferate nor undergo apoptosis in response to estrogen. This makes comparisons to the wild-type T47D cells straightforward.

Because the cells do not show any level of proliferation in response to estrogen, we expect that they will show clear changes in expression of gene(s) or pathways. We do not know whether a single gene or a number of genes is responsible for E_2-ERα-stimulated cell proliferation. This provides several possible explanations of the data. One possibility is that a single gene has been disrupted by the insertion of the luciferase reporter. However, it is equally possible that several genes or pathways are aberrantly regulated in the KBluc cells compared to the T47D WT cells and that in sum these genes are critical for estrogen dependent growth when they are regulated together. It is important to keep in mind that if a gene remains similarly regulated between the two cell lines that this simply indicates that it is not the critical estrogen dependent growth gene in this cell. Therefore, this does not indicate the relative importance of genes concordantly regulated in the two cell lines as they may be critical for estrogen dependent cell proliferation but remain functional. In contrast, the assay comparing effects on gene expression by pM and nM E_2 is a positive assay. Since E_2-ERα-stimulates cell proliferation at both pM and nM E_2, any gene that has lost regulation at pM E_2 is not essential for E_2-ERα-stimulated cell proliferation.

However, this remains a challenging experimental model for a number of technical reasons. One is that E_2-ERα induction of most test mRNAs is much lower than in MCF-7 cells. This will make having an adequate dynamic range, difficult if not impossible on microarrays or RNA-Seq. A possibility that cannot be excluded is that the defect in the KBluc cells is not at the level of transcriptional regulation by ERα but is due to a mutation in an ERα regulated gene and the subsequent loss of downstream effects. Another possibility is that the defect lies at the translational level and involves a nonfunctional protein.

Despite the challenges, the system represents the development of an important new experimental model that has a very specific phenotype. This phenotype can be characterized and explored leading to a greater understanding of genes important in E_2-ERα-stimulated cell proliferation.

4.6 Figures

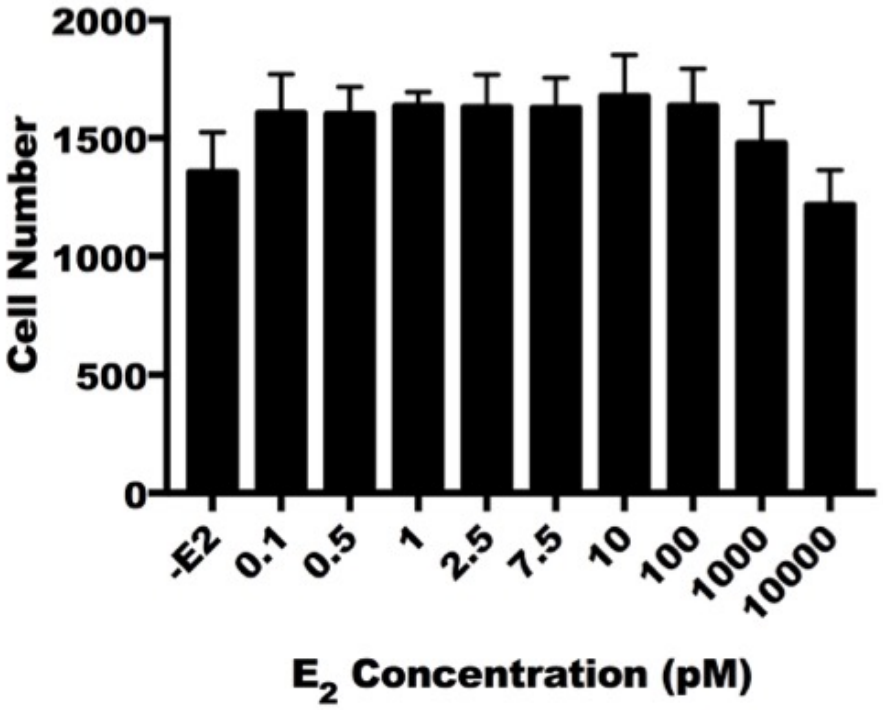

Figure 4.1. T47D-KBluc cells do not proliferate in response to E_2. 1,000 cells/well were plated in a 96 well plate. The indicated concentrations of E_2 in ethanol were added. After 3 days, cell number was determined using MTS and a standard curve of T47D-KBluc cells versus absorbance as we describe (38, 41). Data is the mean $\pm$ SEM for 6 separate sets of cells.

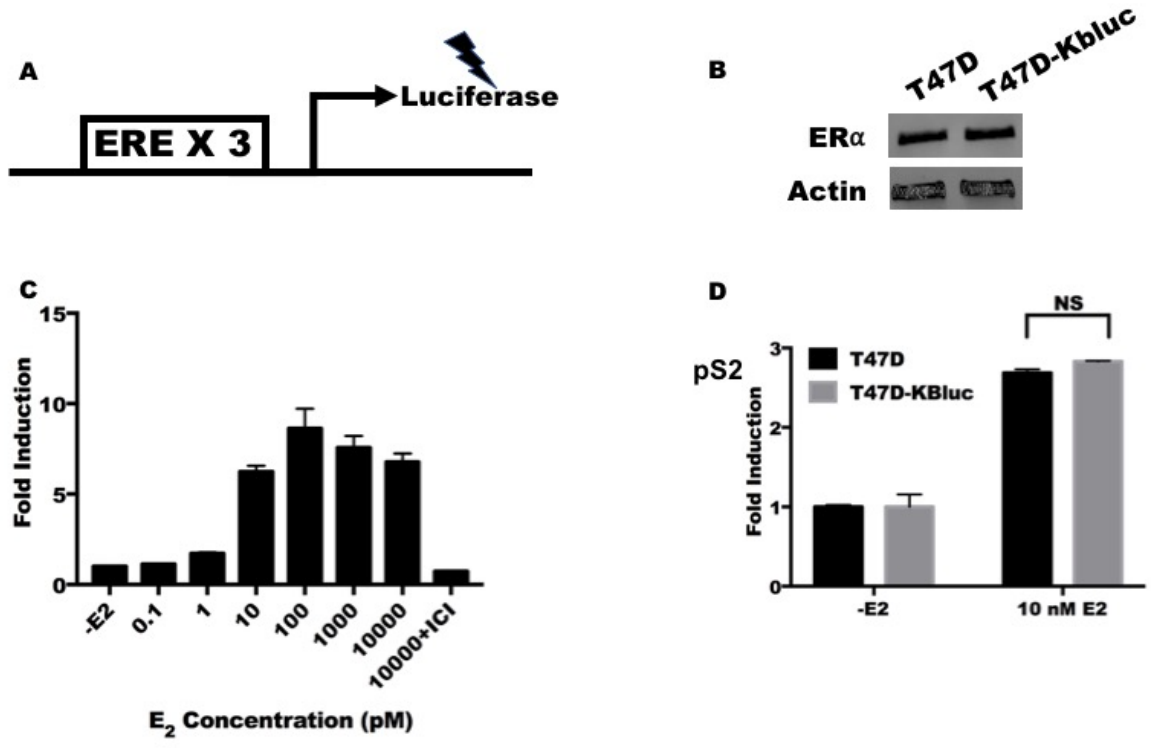

Figure 4.2. KBluc cells contain functional ER. T47D-KBluc cells were maintained in 5% CD-FBS for 4 days prior to being plated in 10% CD-CS (A) Schematic diagram of the KBluc luciferase reporter system. (B) Relative ERα levels in T47D and T47D-KBluc cells. (C) Functional estrogen receptor activates a stably transfected luciferase reporter and is inhibited by the competitive ER antagonist ICI 182 780/Faslodex. (D) qRT PCR data from the endogenously regulated ERα target gene pS2. Data represents mean fold induction over $-E_2$ sample of 3 replicates ± SEM

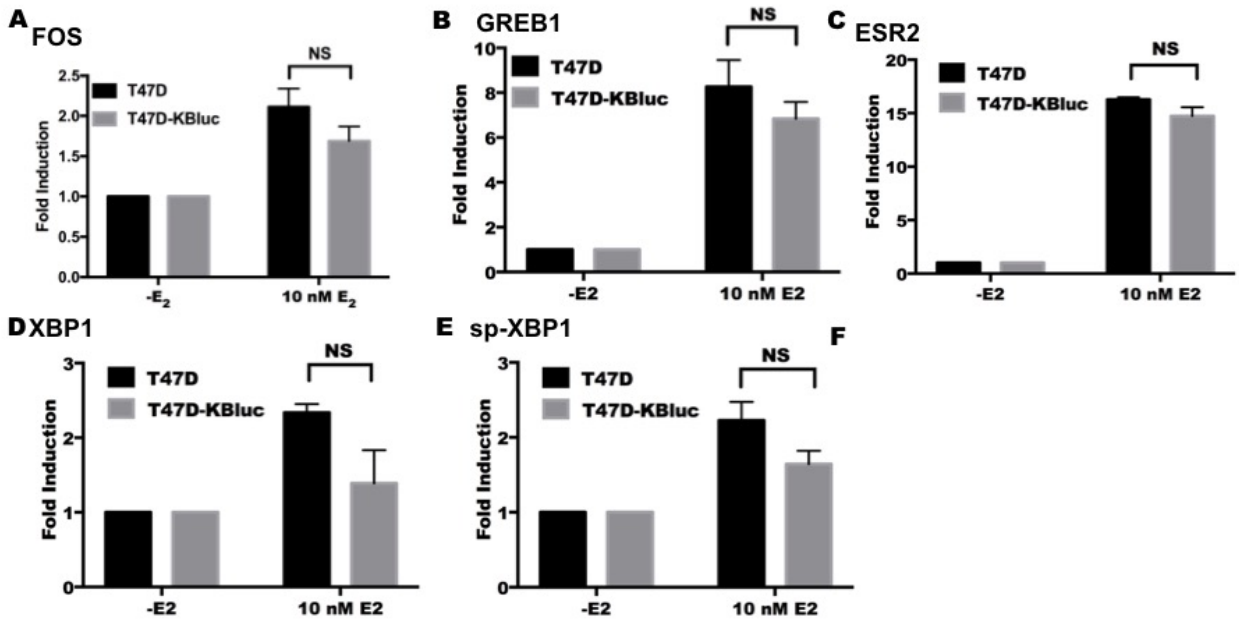

Figure 4.3. Induction of E_2-ERα regulated genes does not differ between T47D and T47D KBluc cells. Wild type T47D cells and KBluc cells were treated for 4 hours with 10 nM E_2, after which mRNA was collected and analyzed via qPCR. Data represents mean fold induction over –E2 sample of 3 replicates ± SEM. (A) FOS (B) GREB1 (C) ESR2 (D)XBP1 (E) sp-XBP1

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Chapter Five

Future Directions

These studies have identified important new tools for the study of ERα and its central role in breast cancer. We demonstrate that what epidemiologic studies have shown regarding circulating levels of estrogen and the risk of breast cancer is biologically plausible. Picomolar concentrations of E_2 can stimulate maximal proliferation of breast cancer cells. We have also shown that these concentrations can regulate the transcription of ERα target genes and found that at least one purported target gene critical for estrogen induced proliferation is not induced at picomolar concentrations. We have also developed a second tool which consists of nearly isogenic cell lines where one cell line contains functional ERα but has a deficiency in estrogen induced cell proliferation. We demonstrate its potential in identifying genes critical for E_2-stimulated cell proliferation.

While we have answered several questions regarding how E_2 functions through ER in breast cancer cells this also leaves many questions and future directions to pursue. One of the largest questions is what are the key E_2-ERα regulated genes? To answer this question future work employs genome wide approaches including microarrays and RNA-Seq. By using escalating concentrations of estrogens one can describe the basal transcriptome of ER up to maximal stimulation. Liqun Yu a graduate student in our lab is starting to answer this question with RNA sequencing studies.

Since only a fraction of the estrogen receptor population is bound with estrogen at picomolar concentration it is important to answer what is the signal that targets the initial mediators of the genome? ChIP-Seq may provide answers by describing the initial binding sites.

This will allow searching for commonalities in the regulated genes and the binding sequences. This would provide a powerful tool to allow us to decode how ER regulates the genome.

The T47D KBluc and T47D cells provide an interesting model where one cell line is derived from the other but emerges with a new phenotype; lack of E_2 induced cell proliferation. Many previously studied systems have introduced exogenous ERα in experimental systems to identify novel regulators of ER induced cell proliferation and have managed provided a wealth of information. However, these systems have difficulty recapitulating the results in systems with endogenous ERα either because the genetic background of the cell lines is so different or because the phenotypes are difficult to distinguish. The KBluc cells provide a plus/minus phenotype in which the cells do not grow in the presence of estrogen, but their wild type counterparts do. These cells provide a new tool to identify critical pathways in E_2-ERα induced growth by looking at discordantly regulated genes on a global level. Lawrence Wang a graduate student in the lab continues to pursue this model and set the system up to look at the entire transcriptome.

In summary, these new tools allow us to explore the ERα transcriptome as it relates to breast cancer cells. Eventually finding the subset of critical downstream targets of ERα will allow for the development of better markers to see if endocrine therapies are functioning, a better understanding of endocrine resistance mechanisms, and a better idea of how to guide therapeutic choice in tumors. Breast cancer affects the lives of nearly a quarter million woman in the US and 1.5 million worldwide. While a number of therapeutic strategies show important initial success, they often lead to resistance. Developing new tools to further our understanding of how estrogen receptor works will guide the development of newer, better therapies.